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**Acute Dermal Irritation Study and  
*Salmonella-Escherichia coli*  
Microsome Plate Incorporation Assay of  
Hydroprocessed Esters and Fatty Acids (HEFA)  
Bio-Based Jet Fuels**

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## PREFACE

Funding for this project was provided through the Alternative Fuels Certification Office (AFLCMC/WNN). This research was conducted under contract FA8650-10-2-6062 with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF). The program manager for the contract was David R. Mattie, PhD (711 HPW/RHDJ), who was also the technical manager for this project.

The dermal irritation study protocol was designed to be in general compliance with the U.S. Environmental Protection Agency (U.S. EPA) Office of Prevention, Pesticides and Toxic Substances (OPPTS) Guideline 870. 2500 (1998a) and the Organisation for Economic Co-operation and Development Guidelines for Testing of Chemicals, Section 404 (OECD, 2002).

The *Salmonella-Escherichia coli*/microsome plate incorporation assay testing procedures were consistent with the OPPTS, Health Effects Test Guidelines, 870.5100 (U.S. EPA, 1998b).

Both studies were conducted in compliance with 40 CFR Part 792, Good Laboratory Practice Standards (GLP).

The dermal irritation study was approved by the Air Force Surgeon General's Office of Research Oversight and Compliance (protocol number AFMC-2011-001A) and the WIL Research Laboratories, LLC, Animal Care and Use Committee (protocol number WIL-773002). The study was conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

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## 1.0 SUMMARY

The U.S. Air Force is developing alternative fuels to decrease dependence on foreign oil. All new fuels are potentially hazardous to Air Force personnel and require toxicity evaluation. The objective of the dermal irritation study was to determine the irritative potential of three biobased jet fuels, Hydroprocessed Esters and Fatty Acids (HEFA)-Camelina (HEFA-C), HEFA-Tallow (HEFA-T) and HEFA-Animal Fats and Oils (HEFA-F), in comparison with the irritative potential of petroleum based JP-8, following a single occluded or semi-occluded exposure to the skin of New Zealand White (NZW) rabbits. Two groups of three male rabbits received a single occluded or semi-occluded exposure to each test substance. Doses (0.5 mL) of each fuel were applied to separate areas of clipped, unabraded skin (four application sites/rabbit). After four hours of exposure, the bandages were removed and the sites washed. Application sites were evaluated in accordance with the method of Draize (1965) and in compliance with U.S. EPA (1998a) at approximately 30 to 60 minutes and 24, 48 and 72 hours after patch removal, and on study days 4, 7 and 14, if irritation persisted.

There were no deaths or remarkable body weight changes noted during the study. Dermal findings during the study consisted of very slight (grade 1) to slight (grade 2) erythema. A score of slightly irritating, as evaluated by the Primary Dermal Irritation Index (PDII) and Descriptive Rating, was determined for the occluded and semi-occluded exposures to all test substances except HEFA-F was found to be non-irritating in the semi-occluded exposure.

The *Salmonella-Escherichia coli*/microsome plate incorporation assay examined two HEFA fuels, HEFA-C and HEFA-T, for mutagenic activity. The assay was performed using the plate incorporation procedure with *S. typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E. coli* strain WP2 (*uvrA*) in both the presence and absence of a metabolic activation mixture (MA) containing an Aroclor 1254-induced rat-liver S9. The range-finding experiment was conducted for both fuels with strain TA100 over doses of 0.156, 0.313, 0.625, 1.25, 2.5 and 5  $\mu$ L/plate (100  $\mu$ L) in the presence and absence of MA containing 5 percent S9. No cytotoxicity was seen at any dose level. The mutagenicity experiments were conducted with all five tester strains at doses of 0.156, 0.313, 0.625, 1.25, 2.5 and 5  $\mu$ L/plate, in the presence and absence of a metabolic activation system containing 5 percent S9 (first experiment) and 10 percent S9 (second experiment).

Slight increases in a number of revertant colonies were determined to be statistically significant ( $p < 0.01$ ); however, revertant colonies were generally less than 2-fold, within the historical range for the strain, not reproducible, and, for the most part, not dose-dependent by regression analysis. Therefore, they were not considered to be a mutagenic response or biologically relevant. Cytotoxicity was not seen under any test condition. HEFA-C and HEFA-T were judged to be non-mutagenic under the test conditions used in this study; therefore, the test substances were determined to be negative in the bacterial reverse mutation assay.

## 2.0 INTRODUCTION

The U.S. Air Force is in the process of developing alternative fuels in order to decrease dependence on foreign oil. Since occupational exposures are potentially hazardous to Air Force personnel, new fuels require evaluation for toxicity. Fischer Tropsch (F-T) fuel was the first alternative jet fuel to be tested and certified for use in the U.S. Air Force fleet. The second class of alternative fuels was initially called hydrotreated renewable jet (HRJ). The HRJ fuels are now referred to as Hydroprocessed Esters and Fatty Acids (HEFA). There are three HEFA fuels being developed and are named based on their starting feedstock: HEFA-C is from camelina oil; HEFA-T is from tallow (rendered beef fat); and HEFA-F is from mixed animal fats and oils. These alternative jet fuels are undergoing toxicological evaluation by the 711 Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Molecular Bioeffect Branch (711 HPW/RHDJ). The results of the toxicity studies of the alternative fuels will be compared with the toxicity of traditional petroleum distillate JP-8 jet fuel. Dermal irritation and mutagenic activity are important studies to evaluate the acute toxicity of a chemical or mixture and are part of the required tests for certification of new fuels (DoD, 2010).

JP-8 was previously evaluated in a skin irritation protocol designed by Draize (1965). Results from this rabbit skin irritation test varied: non-irritating (Smith *et al.*, 1981); slightly irritating (Kinkead *et al.*, 1992); non-irritating (Wolfe *et al.*, 1996); and moderately irritating (Hurley *et al.* (2011). JP-8 is commonly combined with anti-icing and anti-corrosion chemicals, known as the JP-8+100 packages. All JP-8+100 packages were non-irritating when tested for skin irritation (Wolfe *et al.*, 1996). Minor differences in how the studies were conducted may have resulted in the different outcomes. It is also possible that the +100 additives prevented the onset of dermal irritation. In operational use, JP-8 appears to cause dermal irritation to humans based on anecdotal reporting received from operational and medical personnel (Chao *et al.*, 2005). Because of the variable results in past JP-8 animal studies and dermal toxicity seen in humans, it is important to test each new fuel for dermal irritation.

Few dermal systemic toxicity tests, aside from the acute irritation tests discussed above, have been performed with JP-8 itself. A good review of the dermal toxicity of petroleum distillates closely related to JP-8 can be found in McDougal and Rogers (2004). One subchronic dermal study of JP-8 was conducted by Baker and coauthors in 1999. Dermal histological changes were investigated in male F344 rats. A daily un-occluded dermal exposure to 0.156 mL JP-8, JP+100 or JP-4 for four weeks was followed by a three-week recovery period. Proliferative, degenerative and inflammatory changes were significantly greater in the fuel-exposed skin versus non-exposed control skin sites on the same animal immediately post-exposure, but fuel treatment results did not differ from each other. Following the recovery period, the dermal histology of all the exposed skin sites had returned to control scores (Baker *et al.*, 1999). The study herein was designed to evaluate the irritation potential of all three HEFA fuels as well as re-evaluate the irritative potential of JP-8, following a single exposure to the skin of New Zealand White (NZW) albino rabbits.

JP-8 was also previously evaluated for mutagenetic activity in the *Salmonella-Escherichia coli*/microsome plate incorporation assay (Brusick and Matheson, 1978). Microbial mutagenicity assays, developed first by Ames *et al.* (1975), are capable of rapidly detecting the

mutagenic activity of a wide range of chemical classes. Many chemicals that elicit a mutagenic response in the *Salmonella* assay have been shown to be potentially mutagenic and carcinogenic to humans and laboratory animals. One advantage of also using the procedure with *E. coli* is that this strain has an A-T base-pair at the critical mutation site and thus is sensitive to some mutagenic chemicals that are not detected by the *Salmonella* strains. Because microbial mutagenicity assays are short-term, sensitive, and reliable tests for assessing mutagenic potential, their use for genotoxic evaluation of chemicals is appropriate. The study herein was designed to evaluate the mutagenic potential of two HEFA fuels (C and T) in a microbial plate incorporation assay.

## **2.1 Objectives**

The objective of the dermal irritation study was to determine the irritative potential of three biobased jet fuels, HEFA-C, HEFA-T and HEFA-F, in comparison with the irritative potential of petroleum based JP-8 following a single occluded or semi-occluded exposure to the skin of NZW albino rabbits. This study is intended to provide information on the health hazards likely to arise from a short-term exposure to the test substances by the dermal route.

The protocol (Appendix A) was designed to be in general compliance with the U.S. Environmental Protection Agency (EPA) Office of Prevention, Pesticides and Toxic Substances (OPPTS) Guideline 870.2500 (1998a) and the Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals, Section 404 (2002). The study was conducted in compliance with the U.S. EPA Good Laboratory Practices (GLP, 40 CFR Part 792), with the exception that analytical confirmation of the concentration, homogeneity and stability of the fuels was not performed.

The objective of the *Salmonella-Escherichia coli*/microsome plate incorporation assay was to evaluate the ability of two bio-based jet fuels, HEFA-C and HEFA-T, to induce genetic damage as detected by the *Salmonella-E. coli*/microsome assay (Ames test). The protocol can be found in Appendix B. The purpose of this study was to provide data relating to the test substance's health effects, environmental effects, or environmental fate testing regulated by the U.S. EPA. This study, therefore, was conducted in compliance with 40 CFR Part 792 (GLP). Testing procedures were consistent with the OPPTS Health Effects Test Guidelines, 870.5100 (1998b).

## **3.0 METHODS: DERMAL IRRITATION STUDY**

### **3.1 Test Substance Identification**

The test substances were received from 711 HPW/RHDJ, Wright-Patterson Air Force Base OH. Jet fuels were originally provided by AFRL Fuels Branch (RQPF) and are identified by POSF log book numbers assigned by the Fuels Branch. Test substance identification and physical descriptions are located in Table 1.

**Table 1. Test Substance Identification and Physical Descriptions**

<b>Fuel</b>	<b>POSF Lot Number</b>	<b>WIL Log Number</b>	<b>Physical Description</b>
JP-8	POSF 4658	8499A	Clear, light yellow liquid
HEFA-Camelina	POSF 6152	8497A	Clear, colorless liquid
HEFA-Tallow	POSF 6308	8496A	Clear, colorless liquid
HEFA-Animal Fats and Oils	POSF 5469	8498A	Clear, colorless liquid

Purity and stability data were the not the responsibility of WIL Research but was provided by the Air Force. The test substances were stored at room temperature in a flame cabinet and were considered stable under these conditions. A reserve sample of each test substance was collected and stored in the WIL Research Archives.

Prior to use, the original containers of each test substance was inverted to ensure a homogeneous mixture. A vial of each test substance provided by the Air Force was dispensed for dosing.

### **3.2 Test System, Animal Receipt and Acclimation**

New Zealand White albino rabbits were used as the test system on this study. This animal model is generally recognized as appropriate for acute dermal irritation studies. The animals were approximately 46 to 52 weeks old at the initiation of dose administration and a minimum of 2 kg body weight.

Male NZW albino rabbits, in good health, utilized for this study were transferred from the WIL Research acute stock colony on 18 January 2011. The rabbits were weighed and uniquely identified by a plastic ear tag displaying the animal number. The rabbits were acclimated to laboratory conditions for a minimum of five days. During this period, each animal was observed twice daily for mortality and changes in general appearance or behavior.

### **3.3 Animal Care**

Upon arrival, all animals were housed in individual stainless steel cages elevated above ground containing corncob bedding that was changed at least twice weekly. The animals were maintained by the animal husbandry staff of WIL Research in accordance with standard operating procedures (SOPs). The facilities at WIL Research Laboratories, LLC are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

The basal diet used in this study, PMI Nutrition International, LLC (St. Louis MO), Certified High Fiber Rabbit LabDiet® 5325, is a certified feed with appropriate analyses performed by the manufacturer and provided to WIL Research. Municipal water supplying the facility was

analyzed for contaminants according to WIL Research SOPs. The results of the diet and water analyses are maintained at WIL Research. No contaminants were present in animal feed or water at concentrations sufficient to interfere with the objectives of this study. The basal diet was provided at approximately 150 g per day while municipal water, delivered by an automatic watering system, was provided *ad libitum* throughout the acclimation period and during the study.

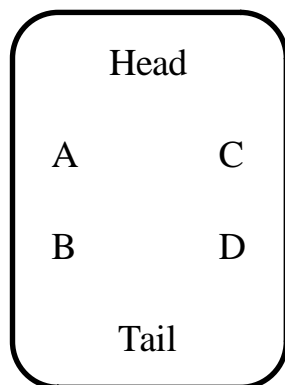
All animals were housed throughout the acclimation period and during the study in an environmentally controlled room. The room temperature and humidity controls were set to maintain environmental conditions of  $66 \pm 5$  °F ( $19 \pm 3$  °C) and  $50 \pm 20$  percent, respectively. Room temperature and relative humidity data were monitored continuously and were scheduled for automatic collection on an hourly basis. These data are summarized in Appendix C. Actual mean daily temperatures ranged from 65.2 °F to 65.9 °F (18.4 °C to 18.8 °C) and mean daily relative humidity levels ranged from 30.6 percent to 53.8 percent during the study. Fluorescent lighting provided illumination for a 12-hour light (0600 hours to 1800 hours) and 12-hour dark photoperiod. The light status (on or off) was recorded once every 15 minutes. Air handling units were set to provide a minimum of ten fresh air changes per hour.

### 3.4 Assignment of Animals to Treatment Groups

Animals used in the study were arbitrarily selected from available stock based upon health and body weight. Body weight values ranged from 3174.6 g to 3972.5 g at initiation of dosing.

### 3.5 Test Substance Administration

There were two groups of three rabbits with four unabraded sites per rabbit (Figure 1). The location of the test sites (designated A through D based upon four available site locations on the back of the rabbit) were rotated so that no test substance was applied to the same site within a group of rabbits. The test sites were delineated with four dots made with indelible ink spaced approximately 2.5 centimeters apart arranged in a square.



**Figure 1. Location of Rabbit Dermal Test Sites**

Each animal received a single, four-hour, occluded or semi-occluded exposure (Groups 1 and 2, respectively) of each test substance on separate areas of intact, unabraded skin. Table 2 presents the study group assignment. No separate control group was utilized; each animal served as its own control.

**Table 2. Study Group Assignment**

<b>Group Number</b>	<b>Test Substances</b>	<b>Dose Volume (mL)*</b>	<b>Exposure Method</b>	<b>Number of Animals</b>
1	JP-8, HEFA-C HEFA-T, HEFA-F	0.5	Occluded	3
2	JP-8, HEFA-C HEFA-T, HEFA-F	0.5	Semi-occluded	3

Note: \*0.5 mL/site, unabraded

On the day prior to dosing, the hair was removed from the backs and flanks of the rabbits using an electric small animal clipper. The clipped area on each animal constituted approximately 20 to 25 percent of the total body surface area. Animals with obvious dermal abnormalities or injuries would have been excluded from the study.

Each 0.5-mL dose was applied to an area of skin approximately 2.5 cm x 2.5 cm under a two-ply gauze patch secured in place with Micropore™ tape (3M, St.Paul MN). For animals in the occluded group, the trunk of the animal was wrapped with plastic wrap. The trunks of animals in both the occluded and semi-occluded group were then wrapped with a gauze binder and secured with Dermiform® tape (Johnson & Johnson, New Brunswick NJ). Plastic restraints (Elizabethan) collars were applied to the animals to prevent ingestion of the test substance and/or bandages. After four hours of exposure, the collars and bandages were removed and each of the sites was wiped with a new disposable paper towel moistened with deionized water. Care was taken not to irritate the skin with the towel.

The selected route of administration for this study was direct application to clipped, unabraded skin (dermal). This route is standard for assessment of local dermal irritative potential. This study was intended to provide information on the health hazards likely to arise from a short-term exposure to the test substances by the dermal route. The experimental design used the procedures and standards required by the current federal and international regulations.



### 3.6 Parameters Evaluated

**3.6.1 Mortality.** The rabbits were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity, including general appearance and behavior. All animals received detailed physical examinations on the day of dosing.

**3.6.2 Body Weights.** Body weights were obtained and recorded on study day 0 (initiation) and at study termination (study day 14).

**3.6.3 Dermal Observations.** The application sites were observed for erythema, edema, and other dermal findings approximately 30 to 60 minutes and 24, 48 and 72 hours after patch removal, and on study days 4, 7 and 14, if irritation persisted. Dermal irritation was graded in accordance with the method of Draize (1965) and in compliance with U.S. EPA (1998a) (see Table 3). The areas of application were clipped free of hair a minimum of one hour prior to scoring, as needed during the study, to facilitate accurate dermal observations.

**Table 3. Evaluation of Dermal Reactions**

Value	Erythema and Eschar Formation
0	No erythema
1	Very slight erythema (barely perceptible, edges of area not well defined)
2	Slight erythema (pale red in color and edges definable)
3	Moderate to severe erythema (definite red in color and area well defined)
4	Severe erythema (beet or crimson red) to slight eschar formation (injuries in depth)
4	Maximum possible erythema score
Value	Edema Formation
0	No edema
1	Very slight edema (barely perceptible, edges of area not well defined)
2	Slight edema (edges of area well defined by definite raising)
3	Moderate edema (raised approximately 1 mm)
4	Severe edema (raised more than 1 mm and extending beyond area of exposure)
4	Maximum possible edema score
8	Maximum total possible Primary Irritation Score

Note: Scoring system from Draize (1965), in compliance with U.S. EPA (1998a)

**3.6.4 Calculation of Primary Dermal Irritation Index.** The Primary Dermal Irritation Index was calculated from scores recorded at 30 to 60 minutes and at 24, 48 and 72 hours after patch removal for the four-hour exposure test sites. The mean scores for erythema and edema were

calculated separately to the nearest tenth and added together. Based on this value, the grading system in Table 4 was used to arrive at the primary dermal irritation descriptive rating.

**Table 4. Descriptive Ratings: Mean Primary Dermal Irritation Index**

<b>Mean Range of Values</b>	<b>Descriptive Rating</b>
0	Nonirritating
0.1 - 2.0	Slightly Irritating
2.1 – 5.0	Moderately Irritating
5.1 – 8.0	Severely Irritating

### 3.7 Termination

After study termination, the rabbits were euthanized by intravenous injection of sodium pentobarbital. The carcasses were discarded without further evaluation.

### 3.8 Data Acquisition and Reporting

The Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF) has title to all documentation records, raw data, or other work product generated during the performance of the study. All remaining work product generated by WIL Research, including raw paper data, are retained in the WIL Research Archives as specified in the study protocol. Archive software systems utilized by WIL are detailed in Table 5.

**Table 5. Archive Software Systems**

<b>Program/System</b>	<b>Description</b>
Archive Management System (AMS)	In-house developed application for storage, maintenance, and retrieval of information for archived materials (e.g., lab books, study data, wet tissues, slides, etc.)
InSight® Publisher	Electronic publishing system (output is Adobe Acrobat, PDF)
Master Schedule	Maintains the master schedule for the company
Metasys DDC Electronic Environmental Control System	Controls and monitors animal room environmental conditions
Microsoft® Office 2002 and 2007; GraphPad Prism® 2008	Used in conjunction with the publishing software to generate study reports
WIL Metasys	In-house developed system used to record and report animal room environmental conditions

Reserve samples of the test substances, pertinent electronic storage media, and the original final report are retained in the WIL Research Archives in compliance with regulatory requirements.

#### 4.0 RESULTS: DERMAL IRRITATION STUDY

There were no deaths during the study. There were no remarkable body weight changes noted during the study (Table 6).

**Table 6. Individual Body Weights**

Group	Number	Sex	Initiation (Day 0, g)	Termination (Day 14, g)
0.5 mL/site, unabraded, occluded	60224	M	3454.6	3453.2
	61517	M	3202.8	3136.0
	60223	M	3972.5	3901.6
0.5 mL/site, unabraded, semi-occluded	61523	M	3174.6	3194.8
	60201	M	3434.0	3482.1
	60222	M	3465.9	3469.3

#### 4.1 Dermal Observations

Dermal findings noted during the study consisted of very slight (grade 1) to slight (grade 2) erythema (Table 7). Individual results are found in Appendix D. Irritation was noted for the occluded and semi-occluded exposures to all test substances, with the exception of the semi-occluded exposure to HEFA-F for which no irritation was noted. Very slight edema was limited to a single occluded exposure to JP-8 on study day 7. Very slight erythema persisted through study day 14 for the occluded exposures to JP-8 (two animals) and HEFA-T (one animal), as well as the semi-occluded exposures to JP-8 (two animals).

**Table 7. Dermal Irritation Scores for JP-8 and Three HEFA Biofuels**

<b>Test Substance</b>	<b>Exposure</b>	<b>PDII</b>	<b>Descriptive Rating</b>
JP-8	Occluded	0.8	Slightly Irritating
	Semi-Occluded	0.8	Slightly Irritating
HEFA-C	Occluded	0.9	Slightly Irritating
	Semi-Occluded	0.6	Slightly Irritating
HEFA-T	Occluded	0.6	Slightly Irritating
	Semi-Occluded	0.2	Slightly Irritating
HEFA-F	Occluded	0.3	Slightly Irritating
	Semi-Occluded	0	Nonirritating

## **4.2 Quality Assurance**

Study signature pages and quality assurance statements are located in Appendix E.

## **5.0 METHODS: *SALMONELLA-ESCHERICHIA COLI*/MICROSOME PLATE INCORPORATION ASSAY**

### **5.1 Design Synopsis**

The route of administration was diluted test article (Table 8) added to an agar containing test system. The plate incorporation method is the standard route to administer a test article to the test system for microbial genotoxicity evaluation of chemicals. The protocol and amendments are presented in Appendix B.

**Table 8. Test and Control Substances**

<b>Test Substances</b>	Names	HEFA-Camelina (POSF6152) HEFA-Tallow (POSF6308)
	Supplier	Air Force Research Laboratory (Wright-Patterson Air Force Base OH)
	Purity	Reported by Sponsor to be greater than 99%
	Physical Descriptions	Clear, colorless liquids
	Storage Conditions	15°–26.5°C
	Characterization	GLP-compliant characterization was not provided by the Sponsor and therefore does not appear in this report
<b>Positive Controls Without Activation</b>		
<b>For Strains TA1535 &amp; TA100</b>	Name	Sodium azide
	CAS Number	26628-22-8
	Manufacturer	Sigma-Aldrich Corp. (St. Louis MO)
	Lot Number	mkb6507v
	Physical Description	White powder
	Storage Conditions	16°–24°C
	Dose/Plate	5 µg/50 µl
<b>For Strain TA1537</b>	Name	9-Aminoacridine hydrochloride hydrate
	CAS Number	52417-22-8
	Manufacturer	Sigma-Aldrich Corp. (St. Louis MO)
	Lot Number	07620td
	Physical Description	Yellow powder
	Storage Conditions	16°–24°C
	Dose/Plate	5 µg/50 µl
<b>For Strain TA98</b>	Name	2-Nitrofluorene
	CAS Number	607-57-8
	Manufacturer	Sigma-Aldrich Corp. (St. Louis MO)
	Lot Number	s43858
	Physical Description	Dull, yellow powder
	Storage Conditions	16°–24°C
	Dose/Plate	5 µg/50 µl
<b>For Strain WP2 (<i>uvrA</i>)</b>	Name	4-Nitroquinoline N-oxide
	CAS Number	56-57-5
	Manufacturer	Sigma-Aldrich Corp. (St. Louis MO)
	Lot Number	090ml161v
	Physical Description	Yellow powder
	Storage Conditions	-19.8° – -14°C
	Dose/Plate	2.5 µg/50 µl

**Table 8. Test and Control Substances (continued)**

<b>Positive Control With Activation</b>	<b>Name</b>	2-Anthramine (2-Aminoanthracene)
	<b>CAS Number</b>	613-13-8
	<b>Manufacturer</b>	Sigma-Aldrich Corp. (St. Louis MO)
	<b>Lot Number</b>	STBB1901
	<b>Physical Description</b>	Green gold powder
	<b>Storage Conditions</b>	16°–24°C
	<b>Dose/Plate</b>	2 µg/50 µL (TA98 and TA100), 4 µg/50 µL (TA1535 and TA1537), and 20 µg/50 µL [WP2 ( <i>uvrA</i> )] in the presence of metabolic activation
	<b>Characterization</b>	Characterization of each positive control article was obtained from the manufacturer's CofA, which is included in Appendix F.
<b>Solvent</b>	<b>Name</b>	Dimethyl sulfoxide (DMSO)
	<b>CAS Number</b>	67-68-5
	<b>Manufacturer</b>	Mallinckrodt Chemicals (Phillipsburg NJ)
	<b>Lot Number</b>	J12J03
	<b>Physical Description</b>	Clear, colorless liquid
	<b>Storage Conditions</b>	15°–26.5°C
	<b>Characterization</b>	Characterization of the solvent was obtained from the manufacturer's Certificate of Analysis (CofA), which is included in Appendix F.
	<b>Preparation of Dose Formulations</b>	An aliquot of each test substance was added to DMSO to make a 0.05 mL/mL stock solution for the range-finding experiment. For the mutagenicity experiments, a stock solution of 0.05 mL/mL was prepared for both test substances. Each stock concentration was mixed on a vortex mixer for two times 60 sec each (range finding experiment for mutagenicity), 2 min (1 <sup>st</sup> experiment for mutagenicity), 1 min (2 <sup>nd</sup> experiment for mutagenicity). In each of the experiments, serial dilutions were made from the initial stock solution and vortexed for 30 sec (range finding, 1 <sup>st</sup> experiment, and 2 <sup>nd</sup> experiment for mutagenicity) between dilutions. Dose formulations were prepared at room temperature, under yellow light, and used on the day they were prepared.
	<b>Characterization of Dose Formulations</b>	Assays to verify the stability, homogeneity, and concentration of each test substance in the vehicle are the responsibility of the Sponsor and are not contained in this final report.
	<b>Disposition</b>	Unused bulk test substance will be returned to the Sponsor. Unused dose formulations, not reserved for dose concentration analysis, were discarded immediately after use in the test system.
	<b>Test Substance Handling</b>	The test substance and dose formulations were handled with the use of eye protection, gloves, and a protective smock or laboratory coat.

## 5.2 Test System

The *Salmonella* tester strains (Table 9) have mutations in the histidine operon, a mutation that leads to a defective lipopolysaccharide coat (*rfa*), and a deletion that covers genes involved in the synthesis of the vitamin biotin (*bio*) and in the repair of ultraviolet (UV)-induced DNA damage (*uvrB*). The *rfa* mutation makes the strains more permeable to many large molecules, thereby increasing the mutagenic effect of these molecules. The *uvrB* mutation renders the bacteria unable to use the accurate excision repair mechanism to remove certain chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents. Strain

TA1535 is reverted to histidine independence by many mutagens that cause base-pair substitutions. TA100 is derived from TA1535 by the introduction of the drug resistance transfer factor, plasmid pKM101. This plasmid is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens (McCann *et al.*, 1975). In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker for detecting the presence of the plasmid in the cell (Mortelmans and Stocker, 1979). The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens such as ICR-191. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. Strain TA98 is derived from TA1538 by adding the plasmid pKM101, which makes it more sensitive to some mutagenic agents (Maron and Ames, 1983; Mortelmans and Zeiger, 2000).

**Table 9. Source of Indicator Organisms**

Species	Strains	Source
<i>Salmonella typhimurium</i> LT2	TA1535, TA1537, TA98, TA100	Dr. Bruce Ames, University of California, Berkeley
<i>Escherichia coli</i>	WP2 ( <i>uvrA</i> )	National Collection of Industrial and Marine Bacteria (NCIMB) (Aberdeen, Scotland)

The *E. coli* WP2 (*uvrA*) strain carries a mutation at the tryptophan (*trp*) allele, which is an auxotrophic mutation reverted by base-pair substitution. The strain is deficient in the repair of UV-induced DNA damage (*uvrA*) (Bridges, 1972; Green and Muriel, 1976; Mortelmans and Riccio, 2000) and thus has enhanced sensitivity to some mutagenic agents.

The strains were analyzed for their genetic markers and for the presence of the plasmid whenever experiments were performed. The indicator strains were kept frozen at -80°C in nutrient broth supplemented with 10 percent sterile glycerol. New frozen stock cultures were made from single colony isolates.

Cultures were inoculated into 50 mL Oxoid Nutrient Broth No. 2 (CM 67) and allowed to sit unshaken for 2 to 4 hours, then gently shaken (125 rpm) for 12 hours at 37 °C. Plates were labeled with indelible ink to identify the test substance, the strain, the dose level, and the presence or absence of the metabolic activation system.

Metabolic activation was induced by an Aroclor 1254-induced rat liver homogenate preparation (S9) supplied by Molecular Toxicology, Inc. (Boone NC) (range-finding and first mutagenicity experiments: Lot No. 2673, 40.70 mg/mL protein; first and second mutagenicity experiments: Lot No. 2708, 41.6 mg/mL protein). The supplier induces liver enzymes of adult male Sprague-Dawley rats by injecting them with Aroclor 1254 (500 mg/kg) five days before they are euthanized. The S9 consists of 9000 × g supernatant of liver homogenized in KCl (1 g wet weight of liver to 3 mL of 0.154 M KCl). Dilutions from each lot of S9, ranging from 0.2 to 10 percent in S9 mix, were tested for their ability to activate benzo(a)pyrene and 2-aminoanthracene to intermediates mutagenic to TA100, prior to product release. The metabolic activation mixture

(Ames *et al.*, 1975; Maron and Ames, 1983) for the experiment(s) consisted of the components and amounts shown in Table 10.

**Table 10. Preparation of Metabolic Activation Mixture for 50 mL Batch**

<b>Ingredient</b>	<b>5% S9 Mix (mL)</b>	<b>10% S9 Mix (mL)</b>
Rat liver S9 (Aroclor 1254-induced)	2.5	5.0
MgCl <sub>2</sub> (0.4 M) and KCl (1.65 M) salts	1.0	1.0
Glucose-6-phosphate (1 M)	0.25	0.25
NADP (0.1 M)	2.0	2.0
Sodium phosphate buffer (0.2 M, pH 7.4)	25.0	25.0
Sterile distilled water	19.25	16.75

### 5.3 Experimental Procedure

To a sterile 13 × 100-mm test tube placed in a 43 °C heating block, the following were added:

1. 2 mL of molten top agar
2. 0.1 mL of indicator organisms (about 10<sup>8</sup> bacteria)
3. appropriate amount of the test substance
4. 0.5 mL of metabolic activation mixture or buffer

This mixture was stirred gently and then poured onto plates containing about 25 mL of minimal glucose agar. For WP2 (*uvrA*), the plates were supplemented with a trace of Oxoid nutrient broth. After the top agar had set, the plates were incubated at approximately 37 °C for about 48 hours. The revertant colonies were counted after the incubation period; however, if the plates could not be immediately evaluated, they were refrigerated at approximately 4 °C for one day until they could be counted.

Concurrent sterility, solvent and positive controls were performed with each experiment. Sterility controls included separately plating out each test substance, metabolic activation mixture, and buffer. Solvent controls were performed for the positive controls and consisted of top agar, bacteria, metabolic activation mixture or buffer, and 50 µL dimethyl sulfoxide (DMSO), the solvent used to dissolve the positive control substances. The solvent control for the test substance, referred to as the zero dose, consisted of top agar, bacteria, metabolic activation mixture or buffer, and DMSO. Positive controls were performed with each strain and consisted of top agar, bacteria, metabolic activation mixture or buffer, and 50 µl of the positive control substance.



## 5.4 Experimental Design

A range-finding experiment was conducted with the test substances to determine a suitable dose range for the mutagenicity experiments. It was performed with *Salmonella* tester strain TA100, in the presence and absence of a metabolic activation mixture (MA) containing 5 percent (volume/volume, v/v) Aroclor 1254-induced rat-liver S9, using three plates per dose level. Dose solutions for the range-finding experiment were achieved by preparing a 0.05 mL/mL (5  $\mu$ L/plate, 100  $\mu$ L dosing volume) stock solution in DMSO of each test substance and serially diluting with DMSO to obtain doses of 2.5, 1.25, 0.625, 0.313 and 0.156  $\mu$ L/plate.

For the mutagenicity experiments, the test substances were assessed in two independent experiments using five tester strains in the presence and absence of metabolic activation, with three plates per dose level. Doses for the mutagenicity experiments with both test substances consisted of 0.156, 0.313, 0.625, 1.25, 2.5, and 5  $\mu$ L/plate and were prepared by serially diluting stock solutions of 0.05 mL/mL in DMSO for each test substance. The test substances were initially tested with and without 5 percent (v/v) S9 in the metabolic activation mix and in the second experiment with 10 percent (v/v) S9.

The highest dose level used in the range-finding experiment was 5  $\mu$ L/plate, the recommended maximum test concentration. Dose selection for the mutagenicity experiments was made to assess the potential dose-response relationship and contained at least three nontoxic dose levels.

## 5.5 Cytotoxicity Assessment

The test plates were compared with the control plates for their revertant count and for the condition of the background bacterial lawn. Toxicity was estimated by several parameters: a substantial decrease in the number of revertant colonies on the test plates, clearing or absence of the background bacterial lawn growth, or formation of pinpoint non-revertant colonies. Endpoints evaluated included the actual numbers of revertant colonies observed on the plates and the condition of the bacterial lawn growth.

## 5.6 Data Collection

Bias was controlled by collecting data with an automated colony counter when possible. The revertant colonies were counted using an automated colony counter. When accurate counts could not be obtained (e.g., because of precipitation on the plates), the colonies were counted manually using an electric probe colony counter. Data were collected using the Sorcerer Image Analysis System (version 2.2), and the Ames Study Manager (version 1.21), made by Perceptive Instruments (Suffolk, England). Counts from the automated colony counter were compared to manual counts prior to collecting data. A complete system calibration is performed annually.

## 5.7 Data Evaluation

An experiment is considered valid when solvent controls are within 10 percent of historical limits for spontaneous revertants, when positive control mutagens elicit a positive response (5-fold increase over the mean value for the solvent for the respective strain), and when there are at least three nontoxic dose levels (mutagenicity experiments). When experimental plates and sterility control plates indicate gross contamination, the results are not considered valid and the experiment is repeated. In addition, whenever experiments are performed, the strains are analyzed to confirm their genetic markers and the presence of the plasmid. If anomalies exist, the experiment is repeated.

Means and standard deviations were calculated from the individual plate counts. Levene's test (Levene, 1960) was performed to determine if a significant difference exists among treatment variances. Treatments were compared with controls using a one-tailed Dunnett's *t*-test (Dunnett, 1980) and within-levels pooled variance. Evaluation of dose-relatedness for all treatments was made by regression analysis (Draper and Smith, 1981) of revertant counts versus the log of the concentrations (to allow inclusion of the zero dose, one was added to the dose before calculating the log). The significance of the regression was tested using a *t*-statistic. The statistical analyses were performed using the SAS analysis system (SAS Institute Inc., Cary NC); the data were read into the SAS program version 9.1 and then the statistical analysis was run on version 6.12.

The following criteria were used as guidelines for the interpretation of the data; however, the conclusions of the study were based upon evaluation and interpretation of the data.

- **Positive.** A test substance is considered a mutagen when a reproducible and statistically significant ( $p < 0.01$ ) increase is observed at one or more dose levels. A statistically significant ( $p < 0.01$ ) dose-related increase in the number of revertants is also considered a positive response.
- **Negative.** A test substance is considered a nonmutagen when the values for the dose levels are not reproducible or significant or when there is no statistically significant dose-related increase in the number of revertants.
- **Inconclusive.** When a test substance cannot be identified clearly as a mutagen or nonmutagen, the results are classified as inconclusive.

## 5.8 Regulatory Compliance

This study was conducted in compliance with 40 CFR Part 792, Good Laboratory Practice Standards (GLP), with the exception that the characterization of the test substances (identity, purity, and stability) and the supporting analytical chemistry of the dose formulations were not provided to the testing facility.

The protocol was amended on 14 February 2011 (Amendment No. 1) to specify the dose levels for the first experiment for mutagenicity, and on 11 March 2011 (Amendment No. 2) to specify the dose levels and S9 concentration to be used in the second experiment for mutagenicity.

All raw data, the original protocol and final report, relevant documents and records specific to this study will be stored at SRI International, 333 Ravenswood Avenue, Menlo Park CA 94025. All records will be maintained for at least 10 years. At the end of the retention period, HJF will be contacted regarding further disposition of these records. Wet specimens (e.g., colonies in agar) and samples of the control articles are not required to be retained.

## **6.0 RESULTS: *SALMONELLA-ESCHERICHIA COLI*/MICROSOME PLATE INCORPORATION ASSAY**

The presence of the appropriate genetic characteristics was verified for the strains used in this study. The results of the controls were acceptable for all experiments (see historical values in Appendix H) as well as the results of the sterility controls (metabolic activation mix, buffer and a dilution of the test substance). There were an adequate number of nontoxic dose levels in the mutagenicity experiments to evaluate the test substance. Therefore, using these criteria, the assay was considered valid.

### **6.1 Range-Finding Experiment**

The range-finding experiment was performed with the two test substances, HEFA-C and HEFA-T, using strain TA100 at doses representing 0.156, 0.313, 0.625, 1.25, 2.5, and 5  $\mu$ L/plate (100  $\mu$ L dosing volume) in the presence and absence of a MA system containing 5 percent S9. The dose formulations at 5  $\mu$ L per plate for both of the test substances appeared to be hazy; no precipitate was seen when they were added to the test system or on the plates when they were counted. Individual and mean plate counts are presented in Appendix G. No dose-related increase in the number of revertant colonies was seen with either test substance. Cytotoxicity was not observed under any test condition.

### **6.2 Mutagenicity Experiments**

The first experiment for mutagenicity was conducted with HEFA-C and HEFA-T using all five tester strains at doses of 0.156, 0.313, 0.625, 1.25, 2.5, and 5  $\mu$ L/plate in the presence and absence of MA containing 5 percent S9. The dose formulations at 5  $\mu$ L/plate again appeared to be hazy. The statistical analyses for the experiment and the individual and mean plate counts are presented in Appendix G. No statistically significant increase in the number of revertant colonies was observed with either test substance, except for slight increases seen with HEFA-C at 0.313  $\mu$ L/plate with TA1537 in the absence of MA, with HEFA-T at 2.5  $\mu$ L/plate with TA1537 in the absence of MA, and HEFA-T at 0.313  $\mu$ L/plate with TA1535 in the presence of MA which were statistically significant ( $p < 0.01$ ) by Dunnett's test. Because these increases were so slight, within the historical range for the strains and not dose-dependent, they were not considered to be a mutagenic response or biologically relevant. Cytotoxicity was not evident under the test conditions.

The second experiment for mutagenicity was conducted with all five tester strains over the same range of doses in the presence and absence of MA containing 10 percent S9. The dose formulations at 5 µL per plate appeared to be hazy. The statistical analyses for the experiment and the individual and mean plate counts are presented in Appendix G. Slight increases in the number of revertant colonies were seen with HEFA-C at 0.313 µL/plate with TA98 in the absence of MA and TA100 at 0.156 µL/plate in the absence of MA. These increases were considered statistically significant ( $p < 0.01$ ) by Dunnett's test; however, because they were so slight, within the historical range for the strain, not reproducible, and not dose-dependent, they were not considered to be a mutagenic response or biologically relevant. Dunnett's test found the slight increases in the number of revertant colonies seen with HEFA-T and TA100 at 0.156 µL/plate in the absence of MA and at doses of 0.156 to 5 µL/plate in the presence of MA to be statistically significant ( $p < 0.01$ ). The increases seen over doses of 0.156 to 5 µL/plate with MA were considered to be statistically significant ( $p < 0.01$ ) by regression analysis. Because the increases observed with HEFA-T were so slight, within the historical range for the strain, and not reproducible (in the absence of MA) they were not considered to be a mutagenic response or biologically relevant. No other statistically significant increases in the number of revertant colonies were observed. No signs of cytotoxicity were seen with either test substance.

## 7.0 DISCUSSION AND CONCLUSIONS

The Primary Dermal Irritation Index (PDII) and Descriptive Rating for occluded exposures to the skin of New Zealand White rabbits resulted in a slightly irritating description for JP-8 and all three HEFA fuels. For semi-occluded exposures, JP-8, HEFA-C and HEFA-T resulted in slightly irritating responses, while HEFA-F was non-irritating.

JP-8 was found to be slightly irritating in a previous study by Kinkead *et al.* (1992). In a more recent study by Hurley *et al.* (2011), JP-8 was also reported as slightly irritating under semi-occluded exposure. However, the occluded exposure resulted in moderate irritation, the highest descriptive rating to date. In the same study, F-T was also slightly irritating under semi-occluded exposure and moderately irritating under occluded exposure. Compared to JP-8 and F-T jet fuels, the HEFA jet fuels appear to be less irritating.

HEFA-C and HEFA-T were judged to be nonmutagenic under the test conditions used in this study; therefore, the test substances were determined to be negative in the bacterial reverse mutation assay.

Brusick and Matheson (1978) demonstrated that JP-8 was not mutagenic using the *Salmonella-Escherichia coli*/microsome plate incorporation assay (Ames test), which was a relatively new. More recently, JP-8 was re-tested for mutagenicity when F-T jet fuels were examined for mutagenic effects. Both JP-8 and F-T jet fuels were negative in the bacterial reverse mutation assay (Mattie *et al.*, 2011; Riccio *et al.*, 2010). To date, each alternative jet fuel tested for mutagenic potential has been shown to be nonmutagenic. Each time petroleum-derived JP-8 has been tested for mutagenicity, the result has been nonmutagenic.

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## APPENDIX A. DERMAL IRRITATION STUDY PROTOCOL



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WIL-773002  
January 14, 2011

### PROTOCOL

#### ACUTE DERMAL IRRITATION STUDY OF HRJ CAMELINA, HRJ TALLOW AND HRJ ANIMAL FATS AND OILS BIO-BASED JET FUELS IN NEW ZEALAND WHITE RABBITS

#### Submitted To:

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## 1 OBJECTIVE:

To determine the irritative potential of the test substances following a single exposure to the skin of albino rabbits.

This protocol has been designed and the study will be conducted in general compliance with the following guidelines:

Environmental Protection Agency (EPA) Office of Prevention, Pesticides and Toxic Substance (OPPTS) guideline 870.2500 (1998).

Organisation for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals, Section 404 (2002).

The European Union (EU) Guideline in the Official Journal of the European Communities [92/69, Annex V, B4 (1992)].

The study will be conducted in compliance with the U.S. EPA Good Laboratory Practices (40 CFR Part 792), with the exception that analytical confirmation of the concentration, homogeneity and stability of the dosing mixture (if prepared) will not be performed.

## 2 PERSONNEL INVOLVED IN THE STUDY:

### 2.1 Sponsor Representative:

David R. Mattie, PhD, DABT  
711 HPW/RHPB  
2729 R Street, Bldg 837  
Wright-Patterson AFB, OH 45433-5707  
Phone: (937) 904-9569  
E-mail: David.Mattie@WPAFB.AF.MIL

### 2.2 WIL Study Director:

Jonathan M. Hurley, BS  
Project Specialist, General Toxicology  
Phone: (419) 289-8700  
Fax: (419) 289-3650  
E-mail: jhurley@wilresearch.com





**2.3 WIL Departmental Responsibilities:**

Teresa D. Morris, BS  
Senior Toxicologist, Toxicology  
Emergency Contact  
Phone: (419) 289-8700  
E-mail: tmorris@wilresearch.com

Mark D. Nemec, BS, DABT  
President and Chief Operating Officer

Jozef J.W.M. Mertens, PhD, DABT  
Senior Director, General Toxicology

Ronald E. Wilson, BS  
Director, Informational Systems

Bennett J. Varsho, MPH, DABT  
Director, Operations

Sally A. Keets, AS  
Senior Operations Manager, Vivarium

Erica L. Lashley, BS, LAT  
Operations Manager, Toxicology

Theresa M. Rafeld, CPhT  
Group Manager, Formulations Laboratory

Carol A. Kopp, BS, LAT  
Manager, Gross Pathology and  
Developmental Toxicology Laboratory

Gwendalyn M. Maginnis, DVM  
Attending Veterinarian

Robert A. Wally, BS  
Manager, Reporting  
and Regulatory Technical Services

Heather L. Johnson, BS, RQAP-GLP  
Manager, Quality Assurance



**3 STUDY SCHEDULE:**

Proposed Experimental Start Date: January 18, 2011  
Proposed Experimental Termination Date: February 1, 2011  
Proposed Audited Draft Report Date: March 15, 2011

**4 TEST SUBSTANCES:**

The Sponsor assumes responsibility for purity and stability determinations (including under test conditions). Information on composition and method of synthesis will be held by the Sponsor.

**4.1 Test Substance #1 Identification / Lot Number:**

JP-8 / POSF4658

**4.2 Test Substance #2 Identification / Lot Number:**

HRJ Camelina/ (UOP) - POSF 6152

**4.3 Test Substance #3 Identification / Lot Number:**

HRJ Tallow/ (UOP) - POSF 6308

**4.4 Test Substance #4 Identification / Lot Number:**

HRJ Animal Fats and Oils/ (Syntroleum) - POSF 5469

**4.5 Purity:**

Responsibility of the Sponsor

**4.6 Stability:**

Considered to be stable for years when properly stored.

**4.7 Physical Descriptions:**

To be documented by WIL Research Laboratories, LLC



**4.8 Storage Conditions:**

Store at room temperature. Keep containers closed tightly. Use and store these materials in cool, dry, well-ventilated areas away from heat, direct sunlight, hot metal surfaces and all sources of ignition.

**4.9 Personnel Safety:**

At minimum, appropriate gloves, eye protection and long sleeves (lab coat) are to be worn during dose administration. Refer to Material Safety Data Sheets for complete available information.

**4.10 Retention Samples:**

Retention samples of the test substances (as received) will be collected in accordance with WIL Research Laboratories, LLC SOP No. T2-001.

**4.11 Unused Test Substances:**

Unused portions of the test substances will be returned following the issuance of the final study report to the contact below.

David R. Mattie, PhD, DABT  
711 HPW/RHPB  
2729 R Street, Bldg 837  
Wright-Patterson AFB, OH 45433-5707  
Phone: (937) 904-9569  
E-mail: David.Mattie@WPAFB.AF.MIL

**5 TEST SYSTEM:****5.1 Species:**

Albino rabbit (*Oryctolagus cuniculus*)

**5.2 Breed:**

New Zealand White

**5.3 Source:**

Covance Research Products, Inc. (USDA License # 23-A-0180)  
(Documentation of the specific breeding facility will be maintained in the study records and included in the final report.)



**5.4 Number on Study:**

Six animals from the acute stock colony

**5.5 Sex:**

Males and/or females (females will be nulliparous and nonpregnant)

**5.6 Body Weight Range:**

2.0 kg or greater

**5.7 Approximate Age:**

Young adult, at least 12 weeks old at initiation of dosing

**5.8 Identification System:**

Each animal will be uniquely identified by a plastic cartag displaying the animal number. Individual cage cards will be affixed to each cage and will display the animal number, group and study number.

**5.9 Justification for Selection:**

This species and breed is generally recognized as appropriate for acute dermal irritation studies. The number of animals selected is the minimum required to satisfy regulatory guidelines. The experimental design uses the procedures and standards required by the current federal and international regulations.

**6 SPECIFIC MAINTENANCE SCHEDULE:****6.1 Animal Housing:**

The animals will be housed individually in stainless steel cages in an environmentally controlled room. Animals will be housed in clean cages elevated above ground corncob bedding or other suitable material that will be changed at least twice each week. Animals will be changed out into clean cages approximately every two weeks. The facilities at WIL Research Laboratories, LLC are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

**6.2 Environmental Conditions:**

Controls will be set to maintain the temperature at  $66 \pm 5^{\circ}\text{F}$  ( $19 \pm 3^{\circ}\text{C}$ ) and the relative humidity at  $50 \pm 20\%$ . Temperature and relative humidity will be monitored continuously. Data for these two parameters will be scheduled for



automatic collection on an hourly basis. Fluorescent lighting controlled by light timers will provide illumination for a 12-hour light/dark photoperiod. Temporary adjustments to the light/dark cycles may be made to accommodate protocol specified activities. The ventilation rate will be set at a minimum of 10 room air changes per hour, 100% fresh air.

### **6.3 Drinking Water:**

Municipal water will be available *ad libitum*. Filters servicing the automatic watering system will be changed regularly according to Standard Operating Procedures (SOPs). Municipal water supplying the laboratory is analyzed for contaminants according to SOPs to ascertain that none are present at concentrations that would be expected to affect the outcome of the study and the results are maintained on file.

### **6.4 Basal Diet:**

PMI Nutrition International, LLC Certified High Fiber Rabbit LabDiet® 5325 will be offered at approximately 150 g/day during the study. The amount of feed provided will be an estimate and will not be documented. Standard Operating Procedures provide specifications for acceptable levels of heavy metals and pesticides that are reasonably expected to be present in the diet without interfering with the purpose or conduct of the study. Analyses are performed and provided by the manufacturer and the results are maintained on file.

## **7 EXPERIMENTAL DESIGN:**

### **7.1 Animal Receipt and Acclimation:**

Each animal was/will be inspected by a qualified technician upon receipt into the acute stock colony. Animals judged to be in good health and suitable as test animals were/will be acclimated to laboratory conditions for a minimum of five days. All animals were/will be weighed initially and permanently identified. During the acclimation period, each animal will be observed twice daily for changes in general appearance and behavior.

All relevant records and data collected during the acclimation period for animals used on this study will be maintained on file.

### **7.2 Veterinary Care:**

Animals will be monitored by the technical staff for any condition requiring possible veterinary care. If any such condition is identified, a staff veterinarian



will be notified for an examination and evaluation. Animals will be treated as outlined in the Animal Welfare Act Compliance section of the protocol.

### **7.3 Route and Rationale of Test Substance Administration:**

The route of administration will be dermal (clipped, intact skin) in order to evaluate the dermal irritation potential of the test substances. This study is intended to provide information on the health hazards likely to arise from a short-term exposure to the test substances by the dermal route.

### **7.4 Organization of Treatment Groups:**

Following the acclimation period, animals will be arbitrarily selected from available stock based upon health and body weight and assigned to 2 groups of 3 rabbits/group as shown below. No separate control group will be utilized; each animal will serve as its own control. The skin of all test sites will be left intact (unabraded).

Group Number	Test Substances*	Dose Volume (mL/Test Substance)	Exposure Method	Number of Animals
1	#1, #2, #3, #4	0.5	Occluded	3
2	#1, #2, #3, #4	0.5	Semi-occluded	3

\*#1 = JP-8; #2 = HRJ Camelina; #3 = HRJ Tallow; #4 = HRJ Animal Fats and Oils

### **7.5 Test Material Preparation:**

The test substances will be administered undiluted as received at a dosage of 0.5 mL. The pH will be determined and recorded.

### **7.6 Animal Preparation:**

On the day prior to dermal applications, the back and flanks of each animal will be clipped free of hair with a small animal clipper. The clipped area on each animal will constitute approximately 20-25% of the total body surface area (actual size of area will not be recorded). Animals with dermal abnormalities or injuries will be excluded.

### **7.7 Method of Administration:**

Four sites located lateral to the midline of the back will be selected on each rabbit. The location of the test sites (designated A-D based upon four available site locations on the back of the rabbit) will be rotated so that no test substance is applied to the same site within a group of rabbits. The test sites will be delineated with four dots made with indelible ink spaced approximately



2.5 centimeters apart arranged in a square. All animals will receive a single application of four test substances.

Each test site will be immediately covered with a two ply, 2.5-cm square gauze patch. The patch will be secured in place with surgical porous tape. For animals in the occluded exposure groups the trunk of the animal will be wrapped with plastic wrap to occlude the test site. The trunk of animals in both the occluded and semi-occluded groups will then be wrapped with gauze bandaging that will be secured with several wrappings of non-irritating tape. Elizabethan collars will be applied to each animal during the exposure period to prevent ingestion of the test substance and/or wrappings.

After the four hours of exposure, the bandages will be removed and residual test substance cleansed from the application sites using clean, disposable paper towels moistened with deionized water (as thoroughly as possible without irritating the skin). The same towel will not be used on more than one site.

## **8 OBSERVATIONS:**

### **8.1 Viability and Clinical Observations:**

All animals will be observed for mortality/moribundity twice daily (morning and afternoon) for the duration of the study. Moribund animals will be removed from study and euthanized by intravenous injection of sodium pentobarbital. All animals will receive a detailed physical examination on the day of dosing.

### **8.2 Dermal Observations:**

Approximately 30-60 minutes after test substance removal, each test site will be examined and the degree of erythema and edema recorded according to the Draize technique (Appendix A). The presence of any other dermal findings will also be recorded. Additional examinations will be performed at approximately 24, 48 and 72 hours after patch removal. If no irritation is present at the 72-hour observation, the study may be terminated.

If irritation is present at the end of 72 hours, additional observations will be performed on days 4, 7 and 14, or until irritation subsides. The study need not normally exceed 14 days after application unless specifically requested and authorized by the Sponsor. Individual animals will be terminated if no irritation is present at the 72-hour or any subsequent observation. At the request of the Sponsor, observations may be terminated prior to 14 days and/or resolution of irritation.



The areas of application will be clipped free of hair a minimum of one hour before scoring, as needed during the study, to facilitate accurate dermal observations.

### **8.3 Body Weights:**

The body weight of each animal will be determined on study day 0 and at termination.

### **8.4 Gross Pathology:**

All animals will be euthanized by intravenous injection of sodium pentobarbital. A gross necropsy examination on major organ systems of the thoracic and visceral cavities will be conducted on all animals found dead or euthanized *in extremis*. Animals euthanized following study termination will be discarded without further examination.

## **9 CALCULATION OF THE PRIMARY DERMAL IRRITATION INDEX:**

The Primary Dermal Irritation Index will be calculated from the scores recorded at 30-60 minutes, 24, 48 and 72 hours (after patch removal). The mean scores for erythema and edema will be calculated separately to the nearest tenth and added together. Based on this value, the grading system in Appendix A will be used to arrive at a primary dermal irritation descriptive rating for each test substance for the occluded and unoccluded method of exposure.

## **10 REPORT:**

The final report will include, but will not necessarily be limited to, the following: compliance statement, summary, objective, test substance identification and receipt information, methods, observations, mortality, body weights, individual and summarized dermal scores/findings, classification of the test substances based on their dermal irritation properties, results and discussion, key personnel, a signed QAU statement and protocol deviation(s), if any.

WIL Research Laboratories will submit one electronic copy (PDF with an MS Word copy of the report text for editing and comments) of an audited draft report in a timely manner upon completion of data collection prior to issuance of the final report. It is expected that the Sponsor will review the draft report and provide comments to WIL within a two-month time frame following submission. Within one month following receipt of the Sponsor's comments, WIL shall provide a revised draft report that incorporates the Sponsor's reasonable revisions and suggestions. One revision will be permitted as part of the cost of the study; additional changes or revisions may be made, at extra cost. WIL will submit the final report within two weeks of receiving authorization from the Sponsor. If the Sponsor's comments and/or authorization to





finalize the report have not been received at WIL within one year following submission of the draft report, WIL may elect to finalize the report following appropriate written notification to the Sponsor. Two electronic copies (PDF) of the final report on CD-R will be provided. Requests for additional paper copies of the final report may result in additional charges.

#### **11 RECORDS TO BE MAINTAINED:**

All original raw data records (as defined by the applicable GLPs and WIL SOPs) generated by WIL Research Laboratories, LLC will be collected and maintained by WIL Research Laboratories, LLC.

#### **12 WORK PRODUCT:**

Sponsor will have title to all documentation records, raw data, specimens, or other work product generated during the performance of the study. All work product including raw paper data, pertinent electronic storage media and specimens will be retained at no charge for a period of six months following issuance of the final report in the Archives at WIL Research Laboratories, LLC. Thereafter, WIL Research Laboratories will charge a monthly archiving fee for retention of all work product. All work product will be stored in compliance with regulatory requirements.

Any work product, including documents, specimens, and samples, that are required by this protocol, its amendments, or other written instructions of the Sponsor, to be shipped by WIL Research Laboratories, LLC to another location will be appropriately packaged and labeled as defined by WIL's SOPs and delivered to a common carrier for shipment. WIL Research Laboratories, LLC will not be responsible for shipment following delivery to the common carrier.

#### **13 QUALITY ASSURANCE:**

The study will be audited by the WIL Quality Assurance Unit while in progress to assure compliance with EPA Good Laboratory Practices and adherence to the protocol and to WIL SOPs. The raw data and draft report will be audited by the WIL Quality Assurance Unit to assure that the final report accurately describes the conduct and the findings of the study.

#### **14 PROTOCOL MODIFICATION:**

Modification of the protocol may be accomplished during the course of this investigation. However, no changes will be made in the study design without the verbal or written permission of the Sponsor. In the event that the Sponsor verbally requests or approves changes in the protocol, such changes will be made by appropriate documentation in the form of protocol amendments. All alterations of the



protocol and reasons for the modification(s) will be signed by the Study Director and the Sponsor Representative.

#### 15 ANIMAL WELFARE ACT COMPLIANCE:

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR). The Sponsor should make particular note of the following:

- The Sponsor signature on this protocol documents for the Study Director the Sponsor's assurance that, for the study described in this protocol, there are no acceptable non-animal alternatives and the study does not unnecessarily duplicate previous experiments.
- Whenever possible, procedures used in this study have been designed to avoid or minimize discomfort, distress or pain to animals. All methods are described in this study protocol or in written laboratory SOPs.
- Animals that experience severe or chronic pain or distress that cannot be relieved will be painlessly euthanized as deemed appropriate by the veterinary staff and Study Director. The Sponsor will be advised by the Study Director of all circumstances which could lead to this action in as timely a manner as possible.



- Methods of euthanasia used during this study are in conformance with the above-referenced regulation.
- The Sponsor/Study Director has considered alternatives to procedures that may cause more than momentary or slight pain or distress to the animals and has provided a written narrative description (AWA covered species) of the methods and sources used to determine that alternatives are not available.

**16 PROTOCOL APPROVAL:**

Sponsor approval received by the Study Director via email on November 15, 2010.

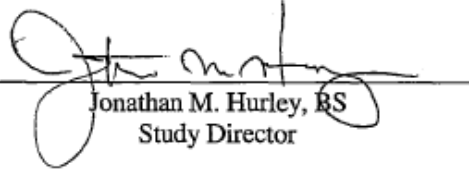
**The Henry M. Jackson Foundation for the Advancement of Military Medicine**



David R. Mattie, PhD, DABT  
Sponsor Representative

1/18/11  
Date

**WIL Research Laboratories, LLC**



Jonathan M. Hurley, BS  
Study Director

1/14/11  
Date



## APPENDIX A

## SCORING CRITERIA FOR DERMAL REACTIONS\*

ValueErythema and Eschar Formation

0	No erythema
1	Very slight erythema (barely perceptible, edges of area not well defined)
2	Slight erythema (pale red in color and edges definable)
3	Moderate to severe erythema (definite red in color and area well defined)
4	Severe erythema (beet or crimson red) to slight eschar formation (injuries in depth)

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4	Maximum possible erythema score
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Edema Formation

0	No edema
1	Very slight edema (barely perceptible, edges of area not well defined)
2	Slight edema (edges of area well defined by definite raising)
3	Moderate edema (raised approximately 1 mm)
4	Severe edema (raised more than 1 mm and extending beyond area of exposure)

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4	Maximum possible edema score
---	------------------------------

8	Maximum total possible Primary Irritation Score
---	---

DESCRIPTIVE RATINGSMean Primary Dermal Irritation Index

<u>Range of Values</u>	<u>Descriptive Rating</u>
0	Nonirritating
0.1 - 2.0	Slightly Irritating
2.1 - 5.0	Moderately Irritating
5.1 - 8.0	Severely Irritating

\*Draize, J.H., 1965. The Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics. Dermal Toxicity, pp. 46-59. Assoc. of Food and Drug Officials of the U.S., Topeka, Kansas and the EPA-OPPTS Health Effects Test Guidelines (1998).



**APPENDIX B. *SALMONELLA-ESCHERICHIA COLI*/MICROSOME PLATE  
INCORPORATION ASSAY PROTOCOL AND AMENDMENTS**



**Evaluation of Two HRJ Fuels in the *Salmonella-Escherichia coli*/Microsome  
Plate Incorporation Assay**

**I. SRI STUDY NUMBER:** G371-11

**II. SPONSOR:** Henry M. Jackson Foundation for the Advancement of  
Military Medicine  
1401 Rockville Pike, Suite 600  
Rockville, MD 20852

**Contract:** Subaward 699531, Prime Award FA8650-10-2-6A02

**Sponsor's Representative:** David R. Mattie, PhD, DABT  
Chief, Nano/Toxicology Section  
711 HPW/RHPBA  
2729 R Street, Bldg 837  
Wright-Patterson Air Force Base, OH 45433-5707  
Phone: 937.904.9569  
Fax: 937.255.1474  
E-mail: [david.mattie@wpafb.af.mil](mailto:david.mattie@wpafb.af.mil)

**III. TESTING FACILITY:** SRI International  
Biosciences Division  
333 Ravenswood Avenue  
Menlo Park, CA 94025-3493

**Study Director:** Edward S. Riccio, BS  
Phone: 650.859.4032  
Fax: 650.859.2889  
E-mail: [edward.riccio@sri.com](mailto:edward.riccio@sri.com)

**Proposed Experimental Start Date:** February 8, 2011  
**Proposed Experimental Termination Date:** March 15, 2011

**IV. APPROVALS:**

David R. Mattie

David R. Mattie, PhD, DABT  
Sponsor's Authorized Representative

31 Jan 11  
Date

Ed S. Riccio

Edward S. Riccio, BS  
SRI Study Director

1/31/2011  
Date

**REVIEWED BY:**

Thomas Bregante  
Thomas Bregante, BS  
SRI Quality Assurance Unit

1/31/2011  
Date

**Evaluation of Two HRJ Fuels in the *Salmonella-Escherichia coli*/  
Microsome Plate Incorporation Assay  
SRI Study No. G371-11**

**V. OBJECTIVE AND PURPOSE OF STUDY**

The objective of this study is to evaluate the ability of two bio-based hydro renewable jet fuels to induce genetic damage as detected by the *Salmonella-E. coli*/microsome assay.

The purpose of this study is to provide data relating to the test substance's health effects, environmental effects, or environmental fate testing regulated by the U.S. Environmental Protection Agency (EPA). This study, therefore, will be conducted in compliance with 40 CFR Part 792, Good Laboratory Practice Standards (GLP). Testing procedures will be consistent with the Office of Prevention, Pesticides and Toxic Substances (OPPTS), Health Effects Test Guidelines, 870.5100.

**VI. MATERIALS AND METHODS**

**A. Experimental Design**

**Route of Administration:** Dissolved/diluted test article added to agar containing test system.

**Reason for Route:** Standard route to administer test article to test system for genotoxic evaluation of chemicals.

**Design.** A range-finding experiment will be conducted with the test substances to determine a suitable dose range for the mutagenicity experiments. The range-finding experiment will be performed with *Salmonella* tester strain TA100, in presence and absence of a rat-liver metabolic activation system (S9), using three plates per dose level, over a wide range of doses. For the mutagenicity experiments, the test substances will be assessed in two experiments using five tester strains in the presence and in the absence of metabolic activation, with three plates per dose level over at least five dose levels (to be added by protocol amendment). The test substances will initially be tested with 5% (v/v) S9 in the S9 mix. If no clear dose-related increase in the number of revertant colonies is observed with a test substance, a 10% (v/v) S9 mix will be used in the repeat experiment. If a mutagenic response is obtained with a test substance in the initial experiment with the 5% S9 mix, the assay will be repeated under the same conditions.

**Justification of Dose Levels Selected.** The highest dose level to be used in the range-finding experiment will be based on solubility or a dose representing 5 µl/plate. Dose selection for the mutagenicity experiments will be made to (1) assess a potential dose-response relationship, (2) include at least one dose that exhibits toxicity, or if a toxic level cannot be achieved, (3) contain a high dose of

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SRI Study No. G371-11**

5 µl/plate, the recommended maximum test concentration for a soluble noncytotoxic test substance.

**Cytotoxicity Assessment.** The test plates will be compared with the control plates for their revertant count and for the condition of the background bacterial lawn. Toxicity is estimated by several parameters: a substantial decrease in the number of revertant colonies on the test plates, clearing or absence of the background bacterial lawn growth, or formation of pinpoint nonrevertant colonies.

**Endpoints Evaluated.** The actual numbers of revertant colonies observed on the plates and the condition of the bacterial lawn growth.

**B. Test and Control Substances**

**1. Test Substances:**

<b>Names / Lot Nos.:</b>	1) HRJ Camelina (UOP) / POSF6152 2) HRJ Tallow (UOP) / POSF6308
<b>Supplier:</b>	Air Force Research Laboratory
<b>Purity:</b>	Reported by Sponsor to be greater than 99%
<b>Physical Descriptions:</b>	To be specified in the final report
<b>Storage Conditions:</b>	Store at room temperature, 15° to 30°C. Keep containers closed tightly. Use and store these materials in cool, dry, well-ventilated areas away from heat, direct sunlight, hot metal surfaces and all sources of ignition.

**Characterization of  
Test Substances:**

Characterization, identity, purity, and stability of the test substances will be the responsibility of the Sponsor and this information will not be contained in the final report.

**Solvent**

<b>Name:</b>	Dimethyl sulfoxide (DMSO)
<b>CAS No.:</b>	67-68-5
<b>Manufacturer:</b>	To be specified in the final report
<b>Lot No.:</b>	To be specified in the final report
<b>Physical Description:</b>	Clear, colorless liquid
<b>Storage Conditions:</b>	Room temperature, 15° to 30°C



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**Characterization of**

**Solvent:**

Characterization of the solvent will be obtained from the manufacturer's Certificate of Analysis.

**Preparation of Dose**

**Formulations:**

An aliquot of the test substance will be prepared in the solvent at a maximum concentration of 0.1 ml/ml. If the test substance is not soluble, it will be gradually diluted until solubility is achieved. Once the stock concentration is prepared, serial dilution will be made from the initial stock. All dose formulations will be prepared at room temperature and mixed thoroughly on a mixing device (at least 5 sec) to ensure homogeneity and adequate solubility. Unless otherwise specified, dose formulations not used on the day of preparation will be stored refrigerated and protected from light. They will be brought to room temperature prior to exposure to the test system.

**Characterization of**

**Dose Formulations:**

Assays to verify the stability, homogeneity, and concentration of each test substance in the vehicle will be the responsibility of the Sponsor and will not be contained in the final report.

**Disposition:**

Unused bulk test substance will be returned to the Sponsor. Unused dose formulations, not reserved for dose concentration analysis, will be discarded immediately after use in the test system.

**Test Substance**

**Handling:**

The test substances and dose formulations will be handled with the use of eye protection, gloves, and a protective smock or laboratory coat.

**Positive Controls without Activation**

**For Strains TA1535 & TA100:** Sodium azide

**CAS No.:** 26628-22-8

**Manufacturer:** To be specified in the final report

**Lot No.:** To be specified in the final report

**Physical Description:** To be specified in the final report

**Storage Conditions:** Room temperature, 15° to 30°C

**Dose/Plate:** 5 µg/50 µl

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<b>For Strain TA1537:</b>	9-Aminoacridine hydrochloride
<b>CAS No.:</b>	52417-22-8
<b>Manufacturer:</b>	To be specified in the final report
<b>Lot No.:</b>	To be specified in the final report
<b>Physical Description:</b>	To be specified in the final report
<b>Storage Conditions:</b>	Room temperature, 15° to 30°C
<b>Dose/Plate:</b>	50 µg/50 µl
<b>For Strain TA98:</b>	2-Nitrofluorene
<b>CAS No.:</b>	607-57-8
<b>Manufacturer:</b>	To be specified in the final report
<b>Lot No.:</b>	To be specified in the final report
<b>Physical Description:</b>	To be specified in the final report
<b>Storage Conditions:</b>	Room temperature, 15° to 30°C
<b>Dose/Plate:</b>	5 µg/50 µl
<b>For Strain WP2 (<i>uvrA</i>):</b>	4-Nitroquinoline N-oxide
<b>CAS No.:</b>	56-57-5
<b>Manufacturer:</b>	To be specified in the final report
<b>Lot No.:</b>	To be specified in the final report
<b>Physical Description:</b>	To be specified in the final report
<b>Storage Conditions:</b>	Frozen, -20° to -10°C
<b>Dose/Plate:</b>	2.5 µg/50 µl
<b><u>Positive Control with Activation</u></b>	
<b>Name:</b>	2-Anthramine (2-Aminoanthracene)
<b>CAS No.:</b>	613-13-8
<b>Manufacturer:</b>	To be specified in the final report
<b>Lot No.:</b>	To be specified in the final report
<b>Physical Description:</b>	To be specified in the final report
<b>Storage Conditions:</b>	Room temperature, 15° to 30°C
<b>Dose/Plate:</b>	2 µg/50 µl (TA98, TA100), 4 µg/50 µl (TA1535, TA1537) & 20 µg/50 µl [WP2 ( <i>uvrA</i> )] in the presence of activation
<b>Characterization of Positive Controls:</b>	Characterization of each positive control substance will be obtained from the manufacturer's Certificate of Analysis.
<b><u>Solvent for the Positive Controls</u></b>	
<b>Name:</b>	Dimethyl sulfoxide (DMSO)
<b>CAS No.:</b>	67-68-5
<b>Manufacturer:</b>	To be specified in the final report
<b>Lot No.:</b>	To be specified in the final report

**Evaluation of Two HRJ Fuels in the *Salmonella-Escherichia coli*/  
Microsome Plate Incorporation Assay  
SRI Study No. G371-11**

<b>Physical Description:</b>	Clear, colorless liquid
<b>Storage Conditions:</b>	Room temperature, 15° to 30°C
<b>Characterization of Solvent:</b>	Characterization of the solvent will be obtained from the manufacturer's Certificate of Analysis.

**C. Test System**

**Test System Justification.** Microbial mutagenicity assays are capable of rapidly detecting the mutagenic activity of many materials, including a wide range of chemical classes. Many chemicals that elicit a mutagenic response in the *Salmonella* assay have been shown to be potentially mutagenic and carcinogenic to humans and laboratory animals. One advantage of using the procedure with *E. coli* is that this strain has an A-T base-pair at the critical mutation site and thus is sensitive to some agents that are not detected by the *Salmonella* strains. Because microbial mutagenicity assays are short-term, sensitive, and reliable tests for assessing mutagenic potential, their use for genotoxic evaluation of chemicals is appropriate.

**Indicator Organisms**

<b>Species:</b>	<i>Salmonella typhimurium</i> LT2
<b>Strains:</b>	TA1535, TA1537, TA98, and TA100
<b>Source:</b>	Dr. Bruce Ames, University of California, Berkeley
<b>Species:</b>	<i>Escherichia coli</i>
<b>Strain:</b>	WP2 ( <i>uvrA</i> )
<b>Source:</b>	National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland

**Description of the Strains.** The *Salmonella* tester strains have mutations in the histidine operon, a mutation that leads to a defective lipopolysaccharide coat (*rfa*), and a deletion that covers genes involved in the synthesis of the vitamin biotin (*bio*) and in the repair of ultraviolet (UV)-induced DNA damage (*uvrB*). The *rfa* mutation makes the strains more permeable to many large molecules, thereby increasing the mutagenic effect of these molecules. The *uvrB* mutation renders the bacteria unable to use the accurate excision repair mechanism to remove certain chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents. Strain TA1535 is reverted to histidine independence by many mutagens that cause base-pair substitutions. TA100 is derived from TA1535 by the introduction of the drug resistance transfer factor, plasmid pKM101. This plasmid is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most

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mutagens (McCann et al., 1975). In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker for detecting the presence of the plasmid in the cell (Mortelmans and Stocker, 1979). The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens such as ICR-191. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. Strain TA98 is derived from TA1538 by adding the plasmid pKM101, which makes it more sensitive to some mutagenic agents (Maron and Ames, 1983; Mortelmans and Zeiger, 2000).

The *E. coli* WP2 (*uvrA*) strain carries a mutation at the tryptophan (*trp*) allele, which is an auxotrophic mutation reverted by base-pair substitution. The strain is deficient in the repair of UV-induced DNA damage (*uvrA*) (Bridges, 1972; Green and Muriel, 1976; Mortelmans and Riccio, 2000) and thus has enhanced sensitivity to some mutagenic agents.

**Test System Identification:** The strains are analyzed for their genetic markers and for the presence of the plasmid whenever experiments are performed.

**Culture Conditions:** The indicator strains are kept frozen at -80°C in nutrient broth supplemented with 10% sterile glycerol. New frozen stock cultures are made from single colony isolates. Cultures are inoculated into 50 ml Oxoid Nutrient Broth No. 2 (CM 67) and allowed to sit unshaken for 2 to 4 hr, then gently shaken (100 to 125 rpm) for about 11 to 14 hr at 37°C.

**Identification:** Plates are labeled with indelible ink to identify the test substance, the strain, the dose level, and the presence or absence of the metabolic activation system.

**Metabolic Activation**  
**Supplier:**

Molecular Toxicology, Inc., Boone, NC

**Description:**

Aroclor 1254-induced rat liver homogenate preparation (S9)

**Preparation:**

Liver enzymes are induced by injecting adult male Sprague-Dawley rats with Aroclor 1254 (500 mg/kg) 5 days before they are sacrificed. The S9 consists of 9000 × g supernatant of liver homogenized in KCl (1 g wet weight of liver to

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3 ml of 0.154M KCl).

**Quality Control:** Dilutions from each lot of S9, ranging from 0.2 to 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene and 2-aminoanthracene to intermediates mutagenic to TA100 prior to product release.

**Metabolic Activation Mixture:** The metabolic activation mixture (Ames et al., 1975; Maron and Ames, 1983) for the experiment(s) will consist of the components and amounts shown below.

**PREPARATION OF METABOLIC ACTIVATION MIXTURE FOR 50 ml**

<u>Ingredient</u>	<u>5% S9 Mix (ml)</u>	<u>10% S9 Mix (ml)</u>
Rat liver S9 (Aroclor 1254-induced)	2.5	5.0
MgCl <sub>2</sub> (0.4 M) and KCl (1.65 M) salts	1.0	1.0
Glucose-6-phosphate (1 M)	0.25	0.25
NADP (0.1 M)	2.0	2.0
Sodium phosphate buffer (0.2 M, pH 7.4)	25.0	25.0
Sterile distilled water	19.25	16.75

**D. Experimental Procedure**

To a sterile 13 × 100-mm test tube placed in a 43°C heating block will be added:

- (1) 2 ml of molten top agar
- (2) 0.1 ml of indicator organisms (about 10<sup>8</sup> bacteria)
- (3) appropriate amount of the test substance
- (4) 0.5 ml of metabolic activation mixture or buffer.

This mixture will be stirred gently, and then poured onto plates containing about



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25 ml of minimal glucose agar. After the top agar has set, the plates will be incubated at ~37°C for about 48 hr. The revertant colonies will be counted after the incubation period; however, if the plates cannot be immediately evaluated, they will be refrigerated at ~4°C until they can be counted.

Concurrent sterility, solvent, and positive controls will be performed with each experiment. Sterility controls will include separately plating out each test substance, metabolic activation mixture, and buffer. Solvent controls will be performed for the positive controls and will consist of top agar, bacteria, metabolic activation mixture or buffer, and 50 µl DMSO, the solvent used to dissolve the positive control substances. The solvent control for the test substance, referred to as the zero dose, will consist of top agar, bacteria, metabolic activation mixture or buffer, and the solvent/diluent for the test substance. Positive controls will be performed with each strain and consist of top agar, bacteria, metabolic activation mixture or buffer, and 50 µl of the positive control substance.

**E. Data Collection**

**Control of Bias.** Bias is controlled by collecting data with an automated colony counter when possible.

**Colony Counting.** The revertant colonies will be counted using an automated colony counter, Sorcerer Image Analysis System (version 2.2) and the data managed through the Ames Study Manager (version 1.21), both manufactured by Perceptive Instruments (Suffolk, England). When accurate counts cannot be obtained (e.g., because of precipitation on the plates), the colonies will be counted manually using an electric probe colony counter.

**F. Evaluation of Data**

**Criteria for Valid Assay.** An experiment is considered valid when solvent controls are within  $\pm 10\%$  of historical limits for spontaneous revertants, when positive control mutagens elicit a positive response ( $\geq 5$ -fold increase over the mean value for the solvent for the respective strain), and when there are at least three nontoxic dose levels (mutagenicity experiments). When experimental plates and sterility control plates indicate gross contamination, the results are not considered valid and the experiment is repeated. In addition, whenever experiments are performed, the strains are analyzed to confirm their genetic markers and the presence of the plasmid. If anomalies exist, the experiment is repeated.

**Statistical Methods.** (1) Means and standard deviation will be calculated from the individual plate counts; (2) Levene's test (Levene, 1960) will be performed to

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determine if a significant difference exists among treatment variances; (3) treatments will be compared with controls by using a one-tailed Dunnett's *t*-test (Dunnett, 1980) and within-levels pooled variance; and (4) evaluation of dose-relatedness for all treatments will be made by regression analysis (Draper and Smith, 1981) of revertant counts versus the log of the concentrations (to allow inclusion of the zero dose, 1 will be added to the dose before calculating the log). The significance of the regression will be tested using a *t*-statistic.

**Criteria for Interpretation.** The following criteria will be used as guidelines for the interpretation of the data; however, the conclusions of the study will be based upon the Study Director's evaluation and interpretation of the data.

**Positive.** A test substance will be considered a mutagen when a reproducible and statistically significant ( $p < 0.01$ ) increase in revertants is observed at one or more dose levels. A statistically significant ( $p < 0.01$ ) dose-related increase in the number of revertants will also be considered a positive response.

**Negative.** A test substance will be considered a nonmutagen when the values for the dose levels are not reproducible or significant or when there is no statistically significant dose-related increase in the number of revertants.

**Inconclusive.** When a test substance cannot be identified clearly as a mutagen or nonmutagen, the results will be classified as inconclusive.

## **VII. REGULATORY COMPLIANCE**

### **A. Good Laboratory Practice (GLP) Compliance**

This study will be conducted in compliance with 40 CFR Part 792, Good Laboratory Practice Standards (GLP), with the exceptions that the characterization of the test substances (identity, purity, and stability) and the supporting analytical chemistry of the dose formulations will not be provided to the testing facility as this testing will not be performed by the Sponsor nor the testing facility.

### **B. Standard Operating Procedures**

All operations pertaining to this study, unless specifically defined in this protocol, will be performed according to the Standard Operating Procedures of the laboratory, and any deviations will be documented.

### **C. Protocol Amendments**

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All changes in or revisions of an approved protocol and the reasons for them will be documented and signed and dated by the Study Director and the Sponsor's Representative. Amendments will be maintained with the protocol. Verbal approval for changes in the protocol may be granted by the Sponsor's Representative, but a written amendment will follow.

**D. Retention of Records and Study Samples**

All raw data, the original protocol and final report, relevant documents, and records specific to this study are the property of the Sponsor and will be stored at SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025. All records will be maintained for at least 10 years. At the end of the retention period, the Sponsor will be contacted regarding further disposition of these records. Wet specimens (e.g., colonies in agar) and samples of the control substances are not required to be retained.

**VIII. REPORTING**

The final report will describe the study design, procedures, and findings and will present an analysis and summary of the data followed by the conclusions derived from the analyses. A draft report will be issued prior to submission of the final report.

**IX. BIBLIOGRAPHY**

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SRI Study No. G371-11

## Protocol Amendment No. 1

**PROTOCOL TITLE:** Evaluation of Two HRJ Fuels in the *Salmonella-Escherichia coli*/Microsome Plate Incorporation Assay

**SRI Study Number:** G371-11

**Sponsor:** Henry M. Jackson Foundation for the Advancement of Military Medicine

**Sponsor's Representative:** David R. Mattie, PhD, DABT

**SRI Study Director:** Edward S. Riccio, BS

This amendment modifies the following lines/sections of the study protocol. Additions are in bold and italics: *addition*. Deleted text has been struck through: ~~deleted~~.

### VI. MATERIALS AND METHODS, A. Experimental Design, Page 2 of 12:

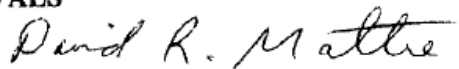
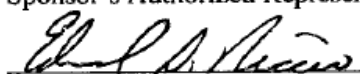
Add to the protocol, the following dose levels to be used for the first experiment for mutagenicity.

*Based on information derived from the range-finding experiment, the first experiment for mutagenicity with HRJ Camelina and HRJ Tallow will be conducted at doses of 0.156, 0.313, 0.625, 1.25, 2.5, and 5 µl/plate in the presence and absence of metabolic activation containing 5% S9.*

**Reason for Change:** This addition to the protocol is necessary to establish the dose levels for the first mutagenicity experiment based on the results of the range-finding experiment.

**Effect on the Study:** These dose levels should allow for the assessment of potential mutagenicity and contain at least three nontoxic dose levels.

#### APPROVALS

	<u>14 Feb 11</u>
David R. Mattie, PhD, DABT	Date
Sponsor's Authorized Representative	
	<u>2/14/2011</u>
Edward S. Riccio	Date
SRI Study Director	

**Protocol Amendment No. 2**

**PROTOCOL TITLE:** Evaluation of Two HRJ Fuels in the *Salmonella-Escherichia coli*/Microsome Plate Incorporation Assay

**SRI Study Number:** G371-11

**Sponsor:** Henry M. Jackson Foundation for the Advancement of Military Medicine

**Sponsor's Representative:** David R. Mattie, PhD, DABT

**SRI Study Director:** Edward S. Riccio, BS

This amendment modifies the following lines/sections of the study protocol. Additions are in bold and italics: *addition*. Deleted text has been struck through: ~~deleted~~.

**VI. MATERIALS AND METHODS, A. Experimental Design, Page 2 of 12:**

Add to the protocol, the following dose levels to be used for the second experiment for mutagenicity.

*Based on information derived from the first experiment for mutagenicity, the second experiment for mutagenicity with HRJ Camelina and HRJ Tallow will be conducted at doses of 0.156, 0.313, 0.625, 1.25, 2.5, and 5 µl/plate in the presence and absence of metabolic activation containing 10% S9.*

**Reason for Change:** This addition to the protocol is necessary to establish the test conditions to be used in the second mutagenicity experiment.

**Effect on the Study:** These dose levels should allow for the assessment of potential mutagenicity and contain at least three nontoxic dose levels.

---

**APPROVALS**

*David R. Mattie*  
\_\_\_\_\_  
David R. Mattie, PhD, DABT  
Sponsor's Authorized Representative

*11 Mar 11*  
\_\_\_\_\_  
Date

*Edward S. Riccio*  
\_\_\_\_\_  
Edward S. Riccio  
SRI Study Director

*3/11/2011*  
\_\_\_\_\_  
Date

# APPENDIX C. DERMAL IRRITATION STUDY ANIMAL ROOM ENVIRONMENTAL CONDITIONS

ACUTE DERMAL STUDY OF BIO-BASED JET FUELS IN RABBITS									
PROJECT NO.:WIL- 773002									
SPONSOR: 773 - H M JACKSON FOUNDATION									
TEMPERATURE/HUMIDITY - STUDY SUMMARY REPORT									
Page 1 of 4									
STUDY SPECIFICATIONS: 773002									
		DATE IN	01/18/11	TIME IN	08:00				
		DATE OUT	02/01/11	TIME OUT	16:00				
ROOM SPECIFICATIONS: B ROOM 106		LOW TEMPERATURE °F: 61.0		HIGH TEMPERATURE °F: 71.0		LOW HUMIDITY %RH: 30.0			
TEST SYSTEM: RABBIT		LOW TEMPERATURE °C: 16.1		HIGH TEMPERATURE °C: 21.7		HIGH HUMIDITY %RH: 70.0			
		SECONDARY TEMP		PRIMARY HUM		SECONDARY HUM			
DATE	MEAN (°F)	MEAN (°C)	MEAN (°F)	MEAN (°C)	MEAN (%RH)	MEAN (%RH)			
01/18/11	65.9	18.8	65.7	18.7	53.8	54.0			
01/19/11	65.7	18.7	65.4	18.6	45.4	45.7			
01/20/11	65.3	18.5	64.8	18.2	42.8	44.3			
01/21/11	65.5	18.6	65.1	18.4	33.5	34.2			
01/22/11	65.6	18.7	65.2	18.4	30.6	31.1			
01/23/11	65.4	18.6	65.1	18.4	31.0	31.4			
01/24/11	65.5	18.6	65.1	18.4	39.3	40.1			
01/25/11	65.5	18.6	65.3	18.5	49.4	50.3			
01/26/11	65.4	18.6	65.2	18.4	47.0	47.7			
01/27/11	65.6	18.7	65.4	18.6	46.5	46.9			
01/28/11	65.6	18.7	65.4	18.6	46.7	47.4			
01/29/11	65.6	18.7	65.2	18.4	46.4	47.1			
01/30/11	65.6	18.7	65.3	18.5	47.0	47.5			
01/31/11	65.8	18.8	65.4	18.6	38.5	38.7			
02/01/11	65.2	18.4	64.8	18.2	45.4	45.6			

ACUTE DERMAL STUDY OF BIO-BASED JET FUELS IN RABBITS  
 TEMPERATURE/HUMIDITY - STUDY SUMMARY REPORT

PROJECT NO.: WIL- 773002  
 SPONSOR: 773 - H M JACKSON FOUNDATION

DATE	PRIMARY TEMP		SECONDARY TEMP		PRIMARY HUM		SECONDARY HUM	
	MEAN (°F)	MEAN (°C)	MEAN (°F)	MEAN (°C)	MEAN (%RH)	MEAN (%RH)	MEAN (%RH)	MEAN (%RH)
<b>SUMMARY OF DAILY MEANS</b>	MEAN	MIN	MAX					
PRIMARY TEMP °F:	65.6	65.2	65.9					
PRIMARY TEMP °C:	18.7	18.4	18.8					
SECONDARY TEMP °F:	65.2	64.8	65.7					
SECONDARY TEMP °C:	18.4	18.2	18.7					
PRIMARY HUM %RH:	42.6	30.6	53.8					
SECONDARY HUM %RH:	43.1	31.1	54.0					
N DAYS	15							

ACUTE DERMAL STUDY OF BIO-BASED JET FUELS IN RABBITS

PROJECT NO.: WIL- 773002

TEMPERATURE/HUMIDITY - STUDY SUMMARY REPORT

SPONSOR: 773 - H M JACKSON FOUNDATION

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**B ROOM 106 SUMMARY OF HOURLY VALUES**

	PRIMARY TEMP		SECONDARY TEMP		PRIMARY HUM	SECONDARY HUM
MEAN	65.6 °F	18.7 °C	65.2 °F	18.4 °C	42.6 %RH	43.1 %RH
MIN	62.4 °F	16.9 °C	62.5 °F	16.9 °C	26.9 %RH	27.3 %RH
MAX	67.5 °F	19.7 °C	66.9 °F	19.4 °C	58.2 %RH	64.9 %RH
SD	0.63	0.35	0.64	0.36	7.36	7.50
SE	0.03	0.02	0.03	0.02	0.40	0.41
N SAMPLES	342		342		341	341
FIRST DAY	01/18/11					
LAST DAY	02/01/11					
N DAYS	15					

**STUDY 773002 SUMMARY OF HOURLY VALUES**

	PRIMARY TEMP	SECONDARY TEMP	PRIMARY HUM	SECONDARY HUM
MEAN	65.6 °F	65.2 °F	42.6 %RH	43.1 %RH
MIN	62.4 °F	62.5 °F	26.9 %RH	27.3 %RH
MAX	67.5 °F	66.9 °F	58.2 %RH	64.9 %RH
SD	0.63	0.64	7.36	7.50
SE	0.03	0.03	0.40	0.41
N SAMPLES	342	342	341	341
FIRST DAY	01/18/11			
LAST DAY	02/01/11			
N DAYS	15			

# APPENDIX D. INDIVIDUAL DERMAL IRRITATION SCORES

INDIVIDUAL DERMAL SCORES													PAGE 1			
Material: JP-8, Occluded																
Site: 0.5 mL/Site (4-Hour Exposure)																
Animal	Sex	Site	Erythema						Edema							
			0.5-1H	24H	48H	72H	4D	7D	14D	0.5-1H	24H	48H		72H	4D	7D
60224-08	M	A	0	1	1	2	2	2	1	0	0	0	0	0	1	0
61517	M	B	0	1	1	1	1	1	1	0	0	0	0	0	0	0
60223-09	M	C	0	1	1	1	1	1	0	0	0	0	0	0	0	0
PII Calculated Using Test Periods: 1H, 24H, 48H, 72H																
Primary Irritation Index (PII) = (0 + 3 + 3 + 4) / 12 + (0 + 0 + 0 + 0) / 12																
PII = 10 / 12 + 0 / 12																
PII = 0.8 + 0																
PII = 0.8 = Slightly Irritating																
M = Male; H = Hours; D = Day																
Site Locations:																
Head																
A C																
B D																
Tail																



INDIVIDUAL DERMAL SCORES

Material: JP-8, Semi-occluded													
Site: 0.5 mL/Site (4-Hour Exposure)													
Animal	Sex	Site	Erythema							Edema			
			0.5-1H	24H	48H	72H	4D	7D	14D	0.5-1H	24H	48H	72H
61523	M	D	0	0	0	1	1	1	1	0	0	0	0
60201-01	M	A	1	1	1	1	2	1	1	0	0	0	0
60222-06	M	B	1	1	1	1	2	1	0	0	0	0	0
PII Calculated Using Test Periods: 1H, 24H, 48H, 72H													
Primary Irritation Index (PII) = $(2 + 2 + 2 + 3) / 12 + (0 + 0 + 0 + 0) / 12$													
PII = $9 / 12 + 0 / 12$													
PII = $0.8 + 0$													
PII = $0.8 =$ Slightly Irritating													
M = Male; H = Hours; D = Day													
Site Locations:													
			Head										
			A	C									
			B	D									
			Tail										

INDIVIDUAL DERMAL SCORES

Material: HRJ Camelina, Occluded																
Site: 0.5 mL/Site (4-Hour Exposure)																
Erythema										Edema						
Animal	Sex	Site	0.5-1H	24H	48H	72H	4D	7D	14D	0.5-1H	24H	48H	72H	4D	7D	14D
60224-08	M	B	1	1	1	1	1	1	1	0	0	0	0	0	0	0
61517	M	C	0	1	1	2	2	1	1	0	0	0	0	0	0	0
60223-09	M	D	0	1	1	1	1	1	0	0	0	0	0	0	0	0
PII Calculated Using Test Periods: 1H, 24H, 48H, 72H																
Primary Irritation Index (PII) = (1 + 3 + 3 + 4) / 12 + (0 + 0 + 0 + 0) / 12																
PII = 11 / 12 + 0 / 12																
PII = 0.9 + 0																
PII = 0.9 = Slightly Irritating																
M = Male; H = Hours; D = Day																

## INDIVIDUAL DERMAL SCORES

Material: HRJ Camelina, Semi-occluded																
Site: 0.5 mL/Site (4-Hour Exposure)																
Group 2			Erythema							Edema						
Animal	Sex	Site	0.5-1H	24H	48H	72H	4D	7D	14D	0.5-1H	24H	48H	72H	4D	7D	14D
61523	M	A	0	1	1	1	1	0	0	0	0	0	0	0	0	0
60201-01	M	B	1	1	1	1	1	0	0	0	0	0	0	0	0	0
60222-06	M	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PII Calculated Using Test Periods: 1H, 24H, 48H, 72H																
Primary Irritation Index (PII) = (1 + 2 + 2 + 2) / 12 + (0 + 0 + 0 + 0) / 12																
PII = 7 / 12 + 0 / 12																
PII = 0.6 + 0																
PII = 0.6 = Slightly Irritating																
M = Male; H = Hours; D = Day																

Material: HRJ Tallow, Occluded																
Site: 0.5 mL/Site (4-Hour Exposure)																
Group 1			Erythema							Edema						
Animal	Sex	Site	0.5-1H	24H	48H	72H	4D	7D	14D	0.5-1H	24H	48H	72H	4D	7D	14D
60224-08	M	C	0	2	2	2	2	1	1	0	0	0	0	0	0	0
61517	M	D	0	1	0	0	0	0	0	0	0	0	0	0	0	0
60223-09	M	A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PII Calculated Using Test Periods: 1H, 24H, 48H, 72H																
Primary Irritation Index (PII) = (0 + 3 + 2 + 2) / 12 + (0 + 0 + 0 + 0) / 12																
PII = 7 / 12 + 0 / 12																
PII = 0.6 + 0																
PII = 0.6 = Slightly Irritating																
M = Male; H = Hours; D = Day																

INDIVIDUAL DERMAL SCORES

Material: HRJ Tallow, Semi-occluded																
Site: 0.5 mL/Site (4-Hour Exposure)																
Group 2			Erythema							Edema						
Animal	Sex	Site	0.5-1H	24H	48H	72H	4D	7D	14D	0.5-1H	24H	48H	72H	4D	7D	14D
61523	M	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60201-01	M	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60222-06	M	D	1	1	0	0	0	0	0	0	0	0	0	0	0	0
PII Calculated Using Test Periods: 1H, 24H, 48H, 72H																
Primary Irritation Index (PII) = (1 + 1 + 0 + 0) / 12 + (0 + 0 + 0 + 0) / 12																
PII = 2 / 12 + 0 / 12																
PII = 0.2 + 0																
PII = 0.2 = Slightly Irritating																
M = Male; H = Hours; D = Day																

## INDIVIDUAL DERMAL SCORES

Material: HRJ Animal Fats and Oils, Occluded																
Site: 0.5 mL/Site (4-Hour Exposure)																
Group 1			Erythema							Edema						
Animal	Sex	Site	0.5-1H	24H	48H	72H	4D	7D	14D	0.5-1H	24H	48H	72H	4D	7D	14D
60224-08	M	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0
61517	M	A	0	1	1	1	1	0	0	0	0	0	0	0	0	0
60223-09	M	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PII Calculated Using Test Periods: 1H, 24H, 48H, 72H																
Primary Irritation Index (PII) = (0 + 1 + 1 + 1) / 12 + (0 + 0 + 0 + 0) / 12																
PII = 3 / 12 + 0 / 12																
PII = 0.3 + 0																
PII = 0.3 = Slightly Irritating																
M = Male; H = Hours; D = Day																
Site Locations:			Head													
			A		C											
			B		D											
			Tail													

INDIVIDUAL DERMAL SCORES

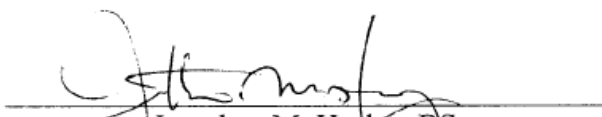
Material: HRJ Animal Fats and Oils, Semi-occluded																
Site: 0.5 mL/Site (4-Hour Exposure)																
Group 2			Erythema							Edema						
Animal	Sex	Site	0.5-1H	24H	48H	72H	4D	7D	14D	0.5-1H	24H	48H	72H	4D	7D	14D
61523	M	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60201-01	M	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60222-06	M	A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PII Calculated Using Test Periods: 1H, 24H, 48H, 72H																
Primary Irritation Index (PII) = (0 + 0 + 0 + 0) / 12 + (0 + 0 + 0 + 0) / 12																
PII = 0 / 12 + 0 / 12																
PII = 0 + 0																
PII = 0 = Nonirritating																
M = Male; H = Hours; D = Day																

## APPENDIX E. SIGNATURE PAGES AND QUALITY ASSURANCE STATEMENTS

### ACUTE DERMAL IRRITATION STUDY OF HRJ CAMELINA, HRJ TALLOW AND HRJ ANIMAL FATS AND OILS BIO-BASED JET FUELS IN NEW ZEALAND WHITE RABBITS

#### Compliance Statement

This study, designated WIL-773002, was conducted in compliance with the U.S. EPA GLP Standards (40 CFR Part 792), 18 September 1989; the WIL Research SOPS; and the protocol as approved by the Sponsor. Analytical confirmation of the concentration, purity, homogeneity, and stability of the test substances was not supplied by the Sponsor and was not conducted as part of this study.

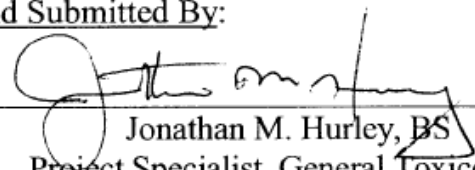
  
Jonathan M. Hurley, BS  
Project Specialist, General Toxicology  
Study Director

01 Apr 2011  
Date




## Report Review and Approval

Approved and Submitted By:

  
Jonathan M. Hurley, BS  
Project Specialist, General Toxicology  
Study Director

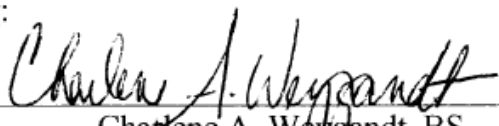
01 Apr 2011  
Date

Report Prepared By:

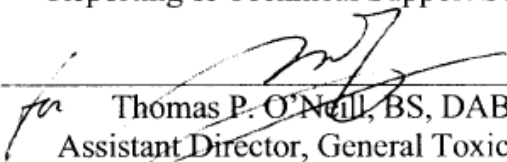
  
Chad P. Durastanti, BS  
Associate Study Analyst

1 April 2011  
Date

Reviewed By:

  
Charlene A. Weygandt, BS  
Lead Analyst and Scientific Advisor,  
Reporting & Technical Support Services

1 April 2011  
Date

  
for Thomas P. O'Neill, BS, DABT  
Assistant Director, General Toxicology

1 Apr. 2011  
Date

## Quality Assurance Unit Statement

### *Phases Inspected*

<u>Date(s) of Inspection(s)</u>	<u>Phase Inspected</u>	<u>Date(s) Findings Reported to Study Director</u>	<u>Date(s) Findings Reported to Management</u>	<u>Auditor(s)</u>
18-Jan-2011 19-Jan-2011	Test Article Administration	19-Jan-2011	28-Feb-2011	R.Rohr
08-Feb-2011	Study Records (Rx-1)	09-Feb-2011	23-Mar-2011	R.Siburt
09-Feb-2011 14-Feb-2011 16-Feb-2011	Study Records (I-1)	16-Feb-2011	23-Mar-2011	R.Siburt
08-Mar-2011 09-Mar-2011 10-Mar-2011	Draft Report	10-Mar-2011	01-Apr-2011	R.Siburt

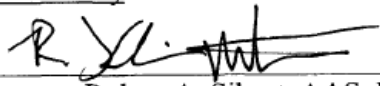
This study was inspected in accordance with the US EPA GLP Standards (40 CFR Part 792), the WIL Research SOPs, and the Sponsor's protocol and protocol amendments. Quality Assurance findings, derived from the inspections during the conduct of the study and from the inspections of the raw data and draft report, are documented and have been reported to the study director. Review of the protocol and protocol amendments (if applicable) as well as a yearly internal facility inspection are conducted by the WIL Research Quality Assurance Unit. A status report is submitted to management monthly.

This report accurately reflects the data generated during the study. The methods and procedures used in the study were those specified in the protocol, its amendments, and the WIL Research SOPs.

*Approval*

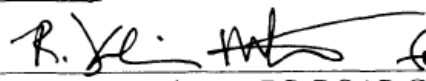
This study was inspected according to the criteria discussed above.

Report Audited By:

 for:  
Robyn A. Siburt, AAS, RLAT  
Compliance Specialist

1 Apr 2011  
Date

Report Released By:


 for:  
Heather L. Johnson, BS, RQAP-GLP  
Manager, Quality Assurance

1 Apr 2011  
Date

**EVALUATION OF TWO HRJ FUELS IN THE *SALMONELLA-ESCHERICHIA COLI*/MICROSOME PLATE INCORPORATION ASSAY**

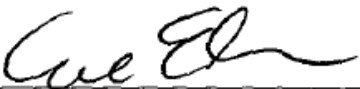
**Report Review and Approval**

Written by:

  
\_\_\_\_\_  
Edward S. Riccio, BS  
Study Director

6/17/2011  
Date

Approved by:

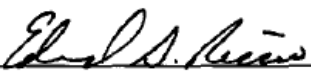
  
\_\_\_\_\_  
Carol E. Green, PhD, DABT  
Senior Director, Toxicology and Pharmacokinetics

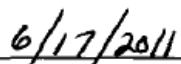
6-17-11  
Date

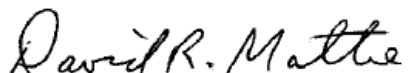
## Good Laboratory Practice Compliance Statement


The study with Two HRJ Fuels, Camelina (UOP) and HRJ Tallow (UOP), submitted by the Sponsor, Henry Jackson Foundation for the Advancement of Military Medicine, was conducted in compliance with the United States Environmental Protection Agency Good Laboratory Practice Standards, 40 CFR Part 792, with the following exceptions:

Characterization of the test substances (identity, purity, and stability) and the supporting analytical chemistry of the dose formulations were not provided to the testing facility as this testing was not performed by the Sponsor or the testing facility.

  
\_\_\_\_\_  
Edward S. Riccio, BS  
Study Director

  
\_\_\_\_\_  
Date

  
\_\_\_\_\_  
David R. Mattie, PhD, DABT  
Sponsor's Representative

  
\_\_\_\_\_  
Date

## Quality Assurance Unit: Final Report and Conflict of Interest Statement

SRI's Quality Assurance Unit assures that the study-- Evaluation of Two HRJ Fuels in the *Salmonella -Escherichia coli*/Microsome Plate Incorporation Assay, SR I Study Number G371-11, has been reviewed for adherence to U. S. Environmental Protection Agency (EPA) under Good Laboratory Practice Standards (40 CFR Part 792).

The following inspections were conducted during this study:

<u>Phase Inspected</u>	<u>Date of Inspection</u>	<u>Date Findings Reported to Management/Study Director</u>
Protocol	01-20-11	01-20-11
Counting	02-17-11	02-17-11
Dose Preparation	03-01-11	03-01-11
Plating	03-16-11	03-16-11
Raw Data	04-27-11	04-27-11
Draft Final Report	04-27-11	04-27-11
Final Report Verification	06-17-11	06-17-11

This statement certifies that the personnel listed below participated in the inspections and audit of this study. These personnel have not been involved in the generation or evaluation of the data. Participation by the individuals listed below poses no conflict of interest.

Beverly Beatty

Noah Fishlock

Jeannette Robinson

Thomas Bregante



Beverly Beatty, RQAP-GLP  
SRI Quality Assurance Unit

06/17/11  
Date


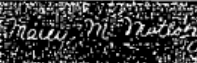
## Key Personnel

<u>Name</u>	<u>Functional Role</u>
Carol E. Green, PhD, DABT	Senior Director, Toxicology and Pharmacokinetics
Mike Hwang, BS	Research Associate
Lisa M. Jack, MA	Statistician
Linh Nguyen, BS	Supervisor, Dose Preparation
Edward S. Riccio, BS	Study Director

## **APPENDIX F. CERTIFICATES OF ANALYSIS**



G371-11

Dimethyl Sulfoxide		
AR <sup>®</sup> (ACS)		
Product No. 4948		
Lot No. J12J03		
Release Date 03/16/2010		
Certificate of Analysis		
TEST	SPECIFICATION	RESULT
Meets A.C.S. Specifications		
Assay ((CH <sub>3</sub> ) <sub>2</sub> SO) (by GC, corrected for water)	99.9 % min.	99.9 %
Appearance (clear, colorless liquid)	Passes Test	Passes Test
Residue after Evaporation	0.01 % max.	< 0.001 %
Titration Acid (meq/g)	0.001 max.	< 0.0002
Water (H <sub>2</sub> O) (by coulometry)	0.1 % max.	0.04 %
For Laboratory, Research or Manufacturing Use		
Country of Origin: USA		
 		
For questions on this Certificate of Analysis please contact Technical Services at 1-800-582-2537 or 908-859-2151 Mallinckrodt Baker, Inc. • 222 Rad School Lane • Phillipsburg, NJ 08865 • Phone: 908.859.2151 • Fax: 908.859.8905		

True copy of original.

Original located: Chemical Recept Binder  
Initials: vr Date: 3/22/11

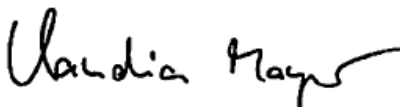
G371-11

# Certificate of Analysis

SIGMA-ALDRICH

Product Name	2-Nitrofluorene, 98%
Product Number	N16754
Product Brand	ALDRICH
CAS Number	607-57-8
Molecular Formula	C <sub>13</sub> H <sub>9</sub> NO <sub>2</sub>
Molecular Weight	211.22

TEST	LOT s43858 RESULTS
QC Acceptance date	14-AUG-2007
APPEARANCE - COLOUR	YELLOW-TAN
APPEARANCE - STATE	POWDER
ELEMENTAL ANALYSIS - CARBON	74.0%
ELEMENTAL ANALYSIS - HYDROGEN	4.3%
ELEMENTAL ANALYSIS - NITROGEN	6.6%
HPLC - PURITY	97.9%
IR SPECTROSCOPY - FTIR SPECTRUM CONFORMS TO STRUCTURE	



Claudia Mayer, Manager  
Quality Control  
Steinheim Germany

G371-11

# Certificate of Analysis

SIGMA-ALDRICH

**Product Name** 4-Nitroquinoline N-oxide  
**Product Number** N8141  
**Product Brand** ALDRICH  
**CAS Number** 56-57-5  
**Molecular Formula** C<sub>9</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub>  
**Molecular Weight** 190.16

## TEST

**Appearance (Color)**  
**Appearance (Form)**  
**Solubility (Turbidity)**

**Solubility (Color)**

**Carbon**

**Nitrogen**

**Purity (HPLC)**

**Specification Date:**

**Date of QC Release:**

**Print Date:**

## SPECIFICATION

Yellow to Brown  
Powder  
Clear to Slightly Hazy  
25 mg/mL, Acetone  
Yellow to Orange  
55.4 - 58.3 %  
14.4 - 15.1 %  
≥98 %

## LOT 090m1161v RESULTS

Yellow  
Powder  
Clear  
Yellow-Orange  
56.9 %  
14.8 %  
99 %  
SEP 2010  
SEP 2010  
SEP 23 2010



Rodney Burbach, Manager  
Quality Control  
St. Louis, Missouri USA

G371-11

# Certificate of Analysis

SIGMA-ALDRICH

Product Name	9-Aminoacridine hydrochloride monohydrate, 98%
Product Number	A38401
Product Brand	ALDRICH
CAS Number	52417-22-8
Molecular Formula	$C_{13}H_{10}N_2 \cdot HCl \cdot H_2O$
Molecular Weight	248.71

TEST	SPECIFICATION	LOT 076201d RESULTS
APPEARANCE	YELLOW OR YELLOW-GREEN POWDER	YELLOW POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE.	CONFORMS TO STRUCTURE.
TITRATION	97.5% - 102.5% (WITH $AgNO_3$ )	98.7% (WITH SILVER NITRATE)
HIGH PRESSURE LIQUID CHROMATOGRAPHY	97.5% (MINIMUM)	99.9%
TITRATION	TYPICALLY 3%-8% $H_2O$ (WITH "KARL FISCHER RGT")	7.0% $H_2O$ (WITH "KARL FISCHER" REAGENT)
PRODUCT CROSS		REPLACES PRODUCT NUMBER A1135
REFERENCE INFORMATION		
QUALITY CONTROL		
ACCEPTANCE DATE		DECEMBER 2005



Barbara Rajzer, Supervisor  
Quality Control  
Milwaukee, Wisconsin USA

9371-11

# Certificate of Analysis

SIGMA-ALDRICH

**Product Name** Sodium azide,  
*ReagentPlus*<sup>®</sup>, ≥99.5%  
**Product Number** S2002  
**Product Brand** SIAL  
**CAS Number** 26628-22-8  
**Molecular Formula** NaN<sub>3</sub>  
**Molecular Weight** 65.01

## TEST

**Appearance (Color)**  
**Appearance (Form)**  
**Purity (Titration by NaOH)**  
**Recommended Retest Period**

## SPECIFICATION

White  
Powder  
≥99.5 %  
\_\_\_\_\_

## LOT mkb6507v RESULTS

White  
Powder  
99.5 %  
\_\_\_\_\_

**Specification Date:**  
**Date of QC Release:**  
**Recommended Retest Date:**  
**Print Date:**

OCT 2010  
OCT 2010  
OCT 2015  
NOV 08 2010

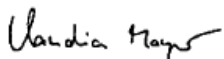


Barbara Rajzer, Supervisor  
Quality Control  
Milwaukee, Wisconsin USA

## Certificate of Analysis

Product Name: 2-AMINOANTHRACENE  
96 %  
Product Number: A38800  
Product Brand: Aldrich  
Molecular Formula:  $C_{14}H_{11}N$   
Molecular Mass: 193.24  
CAS Number: 613-13-8

TEST	SPECIFICATION	LOT STBB1901 RESULTS
APPEARANCE (COLOR)	GOLD TO TAN TO OLIVE-GREEN	OLIVE GREEN
APPEARANCE (FORM)	POWDER	POWDER
PURITY (HPLC AREA %)	≥ 95.5 %	97.5 %
SOLUBILITY (COLOR)	YELLOW TO GREEN TO BROWN	DARK GREEN
SOLUBILITY (TURBIDITY)	CLEAR TO OPAQUE	OPAQUE
SOLUBILITY (METHOD)	50MG/ML DMF	50MG/ML DMF
CARBON CONTENT	83.1 - 91.8 %	86.8 %
NITROGEN CONTENT	6.9 - 7.6 %	7.2 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
QC RELEASE DATE	09/DEC/09	



Claudia Mayer, Manager  
Quality Control  
Steinheim, Germany

Sigma-Aldrich warrants, that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice for additional terms and conditions of sale. The values given on the 'Certificate of Analysis' are the results determined at the time of analysis.

## APPENDIX G. INDIVIDUAL AND MEAN COUNTS - MICROSOME PLATE INCORPORATION ASSAY

**Table 1**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**RANGE-FINDING EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Camelina (UOP)  
Experiment: G371-11 Range Finder  
Assay Conditions: Plate incorporation assay

Study Code: G371-11  
Date Plated: 2/8/2011  
Date Counted: 2/10/2011

### Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	HRJ Camelina	0.156 µl	90.0	8.9	0.8	87 N, 100 N, 83 N
		0.313 µl	100.3	5.7	0.9	105 N, 102 N, 94 N
		0.625 µl	89.0	8.0	0.8	97 N, 81 N, 89 N
		1.25 µl	86.3	8.0	0.8	94 N, 78 N, 87 N
		2.5 µl	84.3	3.1	0.8	81 N, 87 N, 85 N
		5µl	70.7	9.5	0.7	78 N, 60 N, 74 N
	DMSO		108.0	7.2		102, 106, 116
	Untreated Control		128.6	24.2		127, 109, 102, 145, 160
TA100	SA	5 µg	1966.0	17.7	18.2	1950, 1985, 1963
TA100	DMSO	--	105.0	7.0	1.0	105, 98, 112

#### Key to Positive Controls

SA Sodium Azide  
DMSO Dimethyl Sulfoxide

### With metabolic activation (5% S-9)

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	HRJ Camelina	0.156 µl	120.0	9.5	1.0	111 N, 130 N, 119 N
		0.313 µl	116.0	1.0	1.0	117 N, 115 N, 116 N
		0.625 µl	94.3	3.5	0.8	94 N, 98 N, 91 N
		1.25 µl	126.0	15.1	1.1	112 N, 142 N, 124 N
		2.5 µl	122.3	9.0	1.1	128 N, 127 N, 112 N
		5µl	173.3	11.9	1.5	165 N, 168 N, 187 N
	DMSO		115.3	12.5		101, 121, 124
TA100	2AN (5% S9)	2 µg	1606.3	68.9	13.9	1542, 1598, 1679
TA100	DMSO (+S9)	--	124.7	19.5	1.1	111, 116, 147

#### Key to Positive Controls

2AN (5% S9) 2-Aminoanthracene (5% S9)

#### Key to Plate Postfix Codes

N Normal background lawn

**Table 1 (concluded)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**RANGE-FINDING EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Tallow  
Experiment: G371-11 Range Finder  
Assay Conditions: Plate incorporation assay

Study Code: G371-11  
Date Plated: 2/8/2011  
Date Counted: 2/10/2011

**Without metabolic activation**

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	HRJ Tallow	0.156 µl	89.7	4.5	0.8	90 N, 85 N, 94 N
		0.313 µl	111.0	8.5	1.0	112 N, 119 N, 102 N
		0.625 µl	106.3	23.9	1.0	117 N, 123 N, 79 N
		1.25 µl	75.0	10.4	0.7	87 N, 68 N, 70 N
		2.5 µl	79.0	10.4	0.7	74 N, 72 N, 91 N
		5µl	80.3	14.4	0.7	91 N, 64 N, 86 N
	DMSO		108.0	7.2		102 N, 106 N, 116 N
	Untreated Control		128.6	24.2		127, 109, 102, 145, 160
TA100	SA	5 µg	1966.0	17.7	18.2	1950, 1985, 1963
TA100	DMSO	--	105.0	7.0	1.0	105, 98, 112

Key to Positive Controls

SA Sodium Azide  
DMSO Dimethyl Sulfoxide

**With metabolic activation (5% S-9)**

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	HRJ Tallow	0.156 µl	121.3	9.0	1.1	127 N, 126 N, 111 N
		0.313 µl	132.7	7.8	1.2	139 N, 135 N, 124 N
		0.625 µl	104.0	7.5	0.9	96 N, 105 N, 111 N
		1.25 µl	115.3	17.4	1.0	109 N, 102 N, 135 N
		2.5 µl	102.0	2.0	0.9	102 N, 104 N, 100 N
		5µl	122.3	4.2	1.1	127 N, 119 N, 121 N
	DMSO		115.3	12.5		101, 121, 124
TA100	2AN (5% S9)	2 µg	1606.3	68.9	13.9	1542, 1598, 1679
TA100	DMSO (+S9)	--	124.7	19.5	1.1	111, 116, 147

Key to Positive Controls

2AN (5% S9) 2-Aminoanthracene (5% S9)  
DMSO (+S9) Dimethyl Sulfoxide +S9

Key to Plate Postfix Codes

N Normal background lawn



Table 2  
STATISTICAL ANALYSIS OF THE FIRST MUTAGENICITY EXPERIMENT  
WITH TWO HRJ FUELS  
Experiment Performed: 1 March 2011

Test Article	S9 (%)	Dose/ Plate	Mean $\pm$ Standard Deviation Revertants/Plate				WP2uvrA
			TA1535	TA1537	TA98	TA100	
HRJ Camelina (UOP)	0	0 $\mu$ l	15 $\pm$ 3	11 $\pm$ 1	32 $\pm$ 2	142 $\pm$ 1	38 $\pm$ 2
	0	0.156 $\mu$ l	21 $\pm$ 3	13 $\pm$ 4	38 $\pm$ 3	136 $\pm$ 8	21 $\pm$ 2
	0	0.313 $\mu$ l	23 $\pm$ 4*	14 $\pm$ 2	27 $\pm$ 2	145 $\pm$ 9	21 $\pm$ 6
	0	0.625 $\mu$ l	14 $\pm$ 2	12 $\pm$ 2	23 $\pm$ 6	112 $\pm$ 8	25 $\pm$ 2
	0	1.25 $\mu$ l	19 $\pm$ 4	12 $\pm$ 3	24 $\pm$ 4	124 $\pm$ 10	24 $\pm$ 5
	0	2.5 $\mu$ l	13 $\pm$ 2	11 $\pm$ 4	24 $\pm$ 5	145 $\pm$ 7	23 $\pm$ 1
	0	5 $\mu$ l	13 $\pm$ 3	11 $\pm$ 1	27 $\pm$ 4	118 $\pm$ 8	29 $\pm$ 5
ANALYSIS SUMMARY							
Levene's test			N	N	N	N	N
Dose response evaluated via log log regression			N	N	N	N	N
Slope (Standard error)			-8.515 (3.508)	-2.405 (2.077)	-9.174 (4.721)	-17.691 (11.682)	-1.968 (5.613)
Y intercept (Standard error)			19.323 (1.383)	12.805 (0.819)	30.613 (1.861)	136.937 (4.605)	26.486 (2.213)

\* = Significant ( $p < 0.01$ ) by Dunnett's Test

N = Not significant

Table 2 (continued)  
**STATISTICAL ANALYSIS OF THE FIRST MUTAGENICITY EXPERIMENT**  
**WITH TWO HRJ FUELS**  
Experiment Performed: 1 March 2011

Test Article	S9 (%)	Dose/ Plate	Mean $\pm$ Standard Deviation Revertants/Plate			
			TA1535	TA1537	TA98	TA100
HRJ Camelina (UOP)	5	0 $\mu$ l	16 $\pm$ 3	13 $\pm$ 2	40 $\pm$ 5	157 $\pm$ 4
	5	0.156 $\mu$ l	14 $\pm$ 2	11 $\pm$ 1	42 $\pm$ 5	133 $\pm$ 3
	5	0.313 $\mu$ l	12 $\pm$ 0	12 $\pm$ 3	34 $\pm$ 5	127 $\pm$ 17
	5	0.625 $\mu$ l	15 $\pm$ 4	12 $\pm$ 2	36 $\pm$ 6	139 $\pm$ 6
	5	1.25 $\mu$ l	17 $\pm$ 1	11 $\pm$ 1	29 $\pm$ 2	147 $\pm$ 13
	5	2.5 $\mu$ l	10 $\pm$ 2	13 $\pm$ 2	30 $\pm$ 4	150 $\pm$ 6
	5	5 $\mu$ l	16 $\pm$ 3	10 $\pm$ 0	34 $\pm$ 5	143 $\pm$ 9
ANALYSIS SUMMARY						
Levene's test			N	N	N	N
Dose response evaluated via log log regression			N	N	N	N
Slope (Standard error)			-0.767 (2.592)	-2.273 (1.498)	-10.170 (4.652)	6.658 (10.556)
Y intercept (Standard error)			14.465 (1.022)	12.528 (0.591)	38.098 (1.834)	140.463 (4.162)

\* = Significant ( $p < 0.01$ ) by Dunnett's Test

N = Not significant

WP2uvrA  
31  $\pm$  2  
29  $\pm$  4  
37  $\pm$  4  
36  $\pm$  5  
27  $\pm$  4  
33  $\pm$  5  
38  $\pm$  7

Table 2 (continued)  
**STATISTICAL ANALYSIS OF THE FIRST MUTAGENICITY EXPERIMENT**  
**WITH TWO HRJ FUELS**  
**Experiment Performed: 1 March 2011**

Test Article	S9 (%)	Dose/ Plate	Mean $\pm$ Standard Deviation Revertants/Plate			
			TA1535	TA1537	TA98	TA100
HRJ Tallow (UOP)	0	0 $\mu$ l	15 $\pm$ 3	11 $\pm$ 1	32 $\pm$ 2	142 $\pm$ 1
	0	0.156 $\mu$ l	14 $\pm$ 2	12 $\pm$ 1	24 $\pm$ 5	130 $\pm$ 4
	0	0.313 $\mu$ l	14 $\pm$ 1	15 $\pm$ 1	21 $\pm$ 5	134 $\pm$ 4
	0	0.625 $\mu$ l	14 $\pm$ 2	10 $\pm$ 2	22 $\pm$ 1	129 $\pm$ 5
	0	1.25 $\mu$ l	15 $\pm$ 3	14 $\pm$ 4	25 $\pm$ 3	128 $\pm$ 4
	0	2.5 $\mu$ l	20 $\pm$ 4	18 $\pm$ 2*	28 $\pm$ 2	118 $\pm$ 5
	0	5 $\mu$ l	14 $\pm$ 2	12 $\pm$ 2	17 $\pm$ 2	129 $\pm$ 8
						WP2uvrA
<b>ANALYSIS SUMMARY</b>						
Levene's test			N	N	N	N
Dose response evaluated via log log regression			N	N	N	N
Slope (Standard error)			2.446 (2.527)	3.852 (2.560)	-7.898 (4.122)	-15.737 (5.559)
Y intercept (Standard error)			14.421 (0.996)	12.101 (1.009)	26.617 (1.625)	134.598 (2.207)
* = Significant (p< 0.01) by Dunnett's Test						
N = Not significant						

Table 2 (concluded)  
**STATISTICAL ANALYSIS OF THE FIRST MUTAGENICITY EXPERIMENT**  
**WITH TWO HRJ FUELS**  
**Experiment Performed: 1 March 2011**

Test Article	S9 (%)	Dose/ Plate	Mean $\pm$ Standard Deviation Revertants/Plate			
			TA1535	TA1537	TA98	TA100
HRJ Tallow (UOP)	5	0 $\mu$ l	16 $\pm$ 3	13 $\pm$ 2	40 $\pm$ 5	157 $\pm$ 4
	5	0.156 $\mu$ l	16 $\pm$ 3	9 $\pm$ 1	30 $\pm$ 1	146 $\pm$ 4
	5	0.313 $\mu$ l	34 $\pm$ 1*	8 $\pm$ 2	29 $\pm$ 1	149 $\pm$ 6
	5	0.625 $\mu$ l	22 $\pm$ 3	10 $\pm$ 2	35 $\pm$ 3	147 $\pm$ 8
	5	1.25 $\mu$ l	20 $\pm$ 2	8 $\pm$ 2	34 $\pm$ 5	144 $\pm$ 3
	5	2.5 $\mu$ l	16 $\pm$ 3	13 $\pm$ 2	27 $\pm$ 8	149 $\pm$ 2
	5	5 $\mu$ l	16 $\pm$ 2	10 $\pm$ 2	31 $\pm$ 8	133 $\pm$ 6
						WP2uvrA
ANALYSIS SUMMARY						
Levene's test			N	N	N	N
Dose response evaluated via log log regression			N	N	N	N
Slope (Standard error)			-6.942 (5.466)	0.131 (2.044)	-6.346 (4.321)	-19.577 (5.031)
Y intercept (Standard error)			22.002 (2.115)	10.119 (0.806)	34.159 (1.703)	152.351 (1.984)
* = Significant ( $p < 0.01$ ) by Dunnett's Test						
N = Not significant						

Table 3  
STATISTICAL ANALYSIS OF THE SECOND MUTAGENICITY EXPERIMENT  
WITH TWO HRJ FUELS  
Experiment Performed: 16 March 2011

Test Article	S9 (%)	Dose/ Plate	Mean $\pm$ Standard Deviation Revertants/Plate			
			TA1535	TA1537	TA98	TA100
HRJ Camelina (UOP)	0	0 $\mu$ l	14 $\pm$ 2	9 $\pm$ 2	18 $\pm$ 3	117 $\pm$ 2
	0	0.156 $\mu$ l	13 $\pm$ 3	14 $\pm$ 1	25 $\pm$ 5	135 $\pm$ 1*
	0	0.313 $\mu$ l	15 $\pm$ 2	13 $\pm$ 3	27 $\pm$ 3*	120 $\pm$ 4
	0	0.625 $\mu$ l	11 $\pm$ 1	12 $\pm$ 3	26 $\pm$ 1	109 $\pm$ 3
	0	1.25 $\mu$ l	13 $\pm$ 1	15 $\pm$ 2	24 $\pm$ 4	98 $\pm$ 6
	0	2.5 $\mu$ l	11 $\pm$ 3	15 $\pm$ 2	22 $\pm$ 2	85 $\pm$ 3
	0	5 $\mu$ l	13 $\pm$ 2	11 $\pm$ 4	19 $\pm$ 4	82 $\pm$ 2
						WP2uvrA
ANALYSIS SUMMARY						
Levene's test			N	N	N	N
Dose response evaluated via log log regression			N	N	N	N
Slope (Standard error)			-1.373 (2.030)	1.654 (2.570)	-4.543 (3.507)	-62.428 (6.789)
Y intercept (Standard error)			13.024 (0.800)	12.274 (1.013)	24.198 (1.382)	125.001 (2.676)

\* = Significant ( $p < 0.01$ ) by Dunnett's Test

N = Not significant

Table 3 (continued)  
**STATISTICAL ANALYSIS OF THE SECOND MUTAGENICITY EXPERIMENT**  
**WITH TWO HRJ FUELS**  
**Experiment Performed: 16 March 2011**

Test Article	S9 (%)	Dose/ Plate	Mean $\pm$ Standard Deviation Revertants/Plate			
			TA1535	TA1537	TA98	TA100
HRJ Camelina (UOP)	10	0 $\mu$ l	16 $\pm$ 2	13 $\pm$ 1	30 $\pm$ 3	125 $\pm$ 6
	10	0.156 $\mu$ l	10 $\pm$ 2	13 $\pm$ 3	32 $\pm$ 3	114 $\pm$ 11
	10	0.313 $\mu$ l	11 $\pm$ 1	12 $\pm$ 3	40 $\pm$ 6	118 $\pm$ 3
	10	0.625 $\mu$ l	6 $\pm$ 2	12 $\pm$ 3	31 $\pm$ 6	114 $\pm$ 10
	10	1.25 $\mu$ l	8 $\pm$ 2	20 $\pm$ 3	28 $\pm$ 5	110 $\pm$ 8
	10	2.5 $\mu$ l	11 $\pm$ 1	13 $\pm$ 3	30 $\pm$ 0	108 $\pm$ 4
	10	5 $\mu$ l	13 $\pm$ 3	0 $\pm$ 2	34 $\pm$ 3	99 $\pm$ 7
<b>ANALYSIS SUMMARY</b>						
Levene's test			N	N	N	N
Dose response evaluated via log log regression			N	N	N	N
Slope (Standard error)			-0.002 (2.942)	-1.493 (3.014)	-1.177 (4.214)	-27.771 (5.866)
Y intercept (Standard error)			10.763 (1.160)	13.774 (1.188)	32.443 (1.661)	120.771 (2.313)

\* = Significant ( $p < 0.01$ ) by Dunnett's Test

N = Not significant

WP2uvrA

-0.768  
(3.657)

33.703  
(1.442)

Table 3 (continued)  
**STATISTICAL ANALYSIS OF THE SECOND MUTAGENICITY EXPERIMENT**  
**WITH TWO HRJ FUELS**  
Experiment Performed: 16 March 2011

Test Article	S9 (%)	Dose/ Plate	Mean $\pm$ Standard Deviation Revertants/Plate			
			TA1535	TA1537	TA98	TA100
HRJ Tallow (UOP)	0	0 $\mu$ l	14 $\pm$ 2	9 $\pm$ 2	18 $\pm$ 3	117 $\pm$ 2
	0	0.156 $\mu$ l	11 $\pm$ 2	8 $\pm$ 3	20 $\pm$ 2	139 $\pm$ 5*
	0	0.313 $\mu$ l	8 $\pm$ 3	7 $\pm$ 5	21 $\pm$ 4	127 $\pm$ 6
	0	0.625 $\mu$ l	9 $\pm$ 2	10 $\pm$ 2	16 $\pm$ 5	130 $\pm$ 8
	0	1.25 $\mu$ l	6 $\pm$ 1	7 $\pm$ 3	18 $\pm$ 6	115 $\pm$ 4
	0	2.5 $\mu$ l	7 $\pm$ 2	8 $\pm$ 2	21 $\pm$ 6	123 $\pm$ 8
	0	5 $\mu$ l	7 $\pm$ 1	6 $\pm$ 2	15 $\pm$ 1	134 $\pm$ 8
						WP2uvrA
ANALYSIS SUMMARY						
Levene's test			N	N	N	N
Dose response evaluated via log log regression			N	N	N	N
Slope (Standard error)			-6.742 (2.015)	-2.751 (2.120)	-2.637 (3.380)	2.557 (8.447)
Y intercept (Standard error)			10.752 (0.794)	8.622 (0.836)	19.302 (1.333)	125.626 (3.330)

\* = Significant ( $p < 0.01$ ) by Dunnett's Test

N = Not significant



**Table 3 (concluded)**  
**STATISTICAL ANALYSIS OF THE SECOND MUTAGENICITY EXPERIMENT**  
**WITH TWO HRJ FUELS**  
**Experiment Performed: 16 March 2011**

Test Article	S9 (%)	Dose/ Plate	Mean $\pm$ Standard Deviation Revertants/Plate				WP2uvrA
			TA1535	TA1537	TA98	TA100	
HRJ Tallow (UOP)	10	0 $\mu$ l	16 $\pm$ 2	13 $\pm$ 1	30 $\pm$ 3	125 $\pm$ 6	39 $\pm$ 3
	10	0.156 $\mu$ l	11 $\pm$ 2	12 $\pm$ 2	32 $\pm$ 2	154 $\pm$ 2*	25 $\pm$ 2
	10	0.313 $\mu$ l	14 $\pm$ 3	11 $\pm$ 4	27 $\pm$ 4	146 $\pm$ 1*	32 $\pm$ 2
	10	0.625 $\mu$ l	13 $\pm$ 1	10 $\pm$ 0	31 $\pm$ 2	156 $\pm$ 8*	32 $\pm$ 5
	10	1.25 $\mu$ l	15 $\pm$ 2	12 $\pm$ 3	26 $\pm$ 2	155 $\pm$ 7*	29 $\pm$ 3
	10	2.5 $\mu$ l	14 $\pm$ 2	10 $\pm$ 4	29 $\pm$ 3	159 $\pm$ 9*	28 $\pm$ 3
	10	5 $\mu$ l	15 $\pm$ 2	10 $\pm$ 3	30 $\pm$ 2	156 $\pm$ 5*	28 $\pm$ 1
<b>ANALYSIS SUMMARY</b>							
Levene's test			N	N	N	N	N
Dose response evaluated via log log regression			N	N	N	S	N
Slope (Standard error)			1.593 (1.886)	-2.558 (2.101)	-1.138 (2.336)	25.248 (8.638)	-7.218 (3.738)
Y intercept (Standard error)			13.672 (0.744)	11.859 (0.828)	29.479 (0.921)	142.689 (3.405)	32.607 (1.473)

\* = Significant (p< 0.01) by Dunnett's Test

N = Not significant

S = Significant (p<0.01) by specified analyses



**Table 4**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**1<sup>st</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Positive Controls  
Experiment: G371-11 1st Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-11  
Date Plated: 3/1/2011  
Date Counted: 3/4/2011

Without metabolic activation						
Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1535	SA	5 µl	1804.3	27.6	123.0	1802, 1778, 1833
TA1537	9AA	50 µl	584.3	50.8	51.6	643, 554, 556
TA98	2NF	5 µl	1537.3	15.9	48.0	1519, 1548, 1545
TA100	SA	5 µl	2258.0	57.7	15.9	2217, 2324, 2233
WP2uvrA	4NQO	2.5 µl	3291.3	45.6	85.9	3265, 3344, 3265
TA1535	DMSO	--	11.0	1.0	0.8	10, 12, 11
TA1537	DMSO	--	15.7	2.1	1.4	18, 14, 15
TA98	DMSO	--	16.7	3.1	0.5	14, 16, 20
TA100	DMSO	--	138.3	2.5	1.0	141, 136, 138
WP2uvrA	DMSO	--	27.3	2.5	0.7	25, 30, 27
With metabolic activation (5% S9)						
Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1535	2AN (5% S9)	4 µl	246.3	24.5	15.7	222, 271, 246
TA1537	2AN (5% S9)	4 µl	667.0	28.0	50.0	655, 699, 647
TA98	2AN (5% S9)	2 µl	1982.0	180.4	50.0	2172, 1961, 1813
TA100	2AN (5% S9)	2 µl	2879.0	79.6	18.3	2956, 2884, 2797
WP2uvrA	2AN (5% S9)	20 µl	723.7	12.0	23.3	736, 712, 723
TA1535	DMSO (+S9)	--	16.3	2.5	1.0	14, 16, 19
TA1537	DMSO (+S9)	--	16.0	2.6	1.2	15, 19, 14
TA98	DMSO (+S9)	--	33.3	1.5	0.8	33, 35, 32
TA100	DMSO (+S9)	--	152.0	8.9	1.0	149, 145, 162
WP2uvrA	DMSO (+S9)	--	37.3	4.0	1.2	35, 42, 35

Key to Positive Controls

SA Sodium Azide  
9AA 9-Aminoacridine hydrochloride  
2NF 2-Nitrofluorene  
4NQO 4-Nitroquinoline N-oxide  
DMSO Dimethyl Sulfoxide  
2AN (5% S9) 2-Aminoanthracene (5% S9)  
DMSO (+S9) Dimethyl Sulfoxide +S9

Key to Plate Postfix Codes

N Normal background lawn

**Table 4 (continued)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**

**1<sup>st</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Camelina  
Experiment: G371-11 1st Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-11 Camelina  
Date Plated: 3/1/2011  
Date Counted: 3/4/2011

Strain	Compound	Dose level per plate	Without metabolic activation			Individual revertant colony counts
			Mean revertants per plate	Standard Deviation	Ratio treated / solvent	
TA1535	HRJ Camelina	0.156 µl	21.3	3.2	1.5	25, 19, 20
		0.313 µl	23.0	3.6	1.6	20, 22, 27
		0.625 µl	14.3	2.1	1.0	12, 15, 16
		1.25 µl	18.7	4.2	1.3	22, 20, 14
		2.5 µl	12.7	2.1	0.9	15 N, 11 N, 12 N
		5 µl	13.0	2.6	0.9	16 N, 11 N, 12 N
	DMSO		14.7	2.5		15, 17, 12
	Untreated Control		16.0	3.4		15, 14, 19, 12, 20
TA1537	HRJ Camelin	0.156 µl	13.0	4.4	1.1	10, 11, 18
		0.313 µl	14.0	2.0	1.2	12, 14, 16
		0.625 µl	12.3	1.5	1.1	11, 14, 12
		1.25 µl	12.0	3.5	1.1	10, 16, 10
		2.5 µl	11.0	3.6	1.0	10 N, 15 N, 8 N
		5 µl	11.0	1.0	1.0	11 N, 12 N, 10 N
	DMSO		11.3	0.6		12, 11, 11
	Untreated Control		12.6	3.0		12, 18, 11, 11, 11
TA98	HRJ Camelina	0.156 µl	37.7	3.2	1.2	34, 40, 39
		0.313 µl	27.3	2.3	0.9	26, 30, 26
		0.625 µl	22.7	6.4	0.7	30, 20, 18
		1.25 µl	24.3	4.0	0.8	25, 28, 20
		2.5 µl	24.0	4.6	0.8	29 N, 23 N, 20 N
		5 µl	27.3	3.8	0.9	29 N, 23 N, 30 N
	DMSO		32.0	1.7		33, 33, 30
	Untreated Control		29.4	4.3		25, 35, 31, 31, 25
TA100	HRJ Camelina	0.156 µl	136.3	7.5	1.0	132, 132, 145
		0.313 µl	145.0	9.0	1.0	154, 145, 136
		0.625 µl	112.0	7.5	0.8	113, 104, 119
		1.25 µl	124.0	10.4	0.9	119, 117, 136
		2.5 µl	145.0	7.2	1.0	139 N, 143 N, 153 N
		5 µl	118.0	7.9	0.8	124 N, 121 N, 109 N
	DMSO		141.7	0.6		141, 142, 142
	Untreated Control		128.2	8.9		121, 141, 128, 132, 119
WP2uvrA	HRJ Camelina	0.156 µl	23.7	3.5	0.6	27, 20, 24
		0.313 µl	18.3	3.5	0.5	18, 22, 15
		0.625 µl	25.7	2.3	0.7	27, 27, 23
		1.25 µl	27.0	4.4	0.7	30, 29, 22
		2.5 µl	23.7	2.1	0.6	26 N, 22 N, 23 N
		5 µl	31.3	1.5	0.8	30 N, 31 N, 33 N
	DMSO		38.3	2.3		41, 37, 37
	Untreated Control		40.2	6.3		38, 40, 46, 31, 46
						Key to Plate Postfix Codes
						N Normal background lawn

**Table 4 (continued)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**1<sup>st</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Camelina  
Experiment: G371-11 1st Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-11  
Date Plated: 3/1/2011  
Date Counted: 3/4/2011

With metabolic activation (5% S9)						
Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1535	HRJ Camelina	0.156 µl	14.3	2.1	0.9	12, 16, 15
		0.313 µl	12.0	0.0	0.8	12, 12, 12
		0.625 µl	15.3	3.5	1.0	15, 12, 19
		1.25 µl	16.7	1.2	1.1	16, 16, 18
		2.5 µl	10.0	1.7	0.6	11 N, 8 N, 11 N
		5 µl	15.7	3.1	1.0	15 N, 19 N, 13 N
	DMSO		15.7	3.2		17, 18, 12
TA1537	HRJ Camelina	0.156 µl	11.0	1.0	0.8	10, 11, 12
		0.313 µl	12.3	3.2	0.9	10, 11, 16
		0.625 µl	12.3	1.5	0.9	14, 11, 12
		1.25 µl	11.3	0.6	0.9	11, 11, 12
		2.5 µl	12.7	2.1	1.0	15 N, 12 N, 11 N
		5 µl	10.0	0.0	0.8	10 N, 10 N, 10 N
	DMSO		13.3	2.1		14, 15, 11
TA98	HRJ Camelina	0.156 µl	42.3	4.7	1.1	46, 44, 37
		0.313 µl	34.0	5.2	0.9	40, 31, 31
		0.625 µl	36.3	6.0	0.9	30, 42, 37
		1.25 µl	28.7	1.5	0.7	30, 27, 29
		2.5 µl	30.3	4.2	0.8	27 N, 35 N, 29 N
		5 µl	34.3	5.1	0.9	40 N, 33 N, 30 N
	DMSO		39.7	5.1		44, 34, 41
TA100	HRJ Camelina	0.156 µl	133.0	2.6	0.8	131, 132, 136
		0.313 µl	127.3	16.8	0.8	108, 138, 136
		0.625 µl	139.3	5.8	0.9	136, 146, 136
		1.25 µl	147.3	12.7	0.9	139, 141, 162
		2.5 µl	150.3	5.5	1.0	150 N, 156 N, 145 N
		5 µl	142.7	9.0	0.9	152 N, 134 N, 142 N
	DMSO		157.0	4.0		153, 157, 161
WP2uvrA	HRJ Camelina	0.156 µl	29.0	4.0	0.9	33, 29, 25
		0.313 µl	37.0	4.4	1.2	34, 42, 35
		0.625 µl	36.0	5.2	1.2	33, 42, 33
		1.25 µl	26.7	4.0	0.9	29, 22, 29
		2.5 µl	33.0	5.3	1.1	37 N, 35 N, 27 N
		5 µl	38.3	6.7	1.2	31 N, 44 N, 40 N
	DMSO		31.0	2.0		33, 29, 31
Key to Plate Postfix Codes						
N						Normal background lawn

**Table 4 (continued)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**1<sup>st</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Tallow  
Experiment: G371-11 1st Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-11  
Date Plated: 3/1/2011  
Date Counted: 3/4/2011

Strain	Compound	Dose level per plate	Without metabolic activation			Individual revertant colony counts
			Mean revertants per plate	Standard Deviation	Ratio treated / solvent	
TA1535	HRJ Tallow	0.156 µl	14.0	2.0	1.0	14, 12, 16
		0.313 µl	14.3	0.6	1.0	15, 14, 14
		0.625 µl	14.3	2.1	1.0	15, 16, 12
		1.25 µl	14.7	3.1	1.0	12, 18, 14
		2.5 µl	20.3	4.0	1.4	18 N, 25 N, 18 N
		5 µl	13.7	1.5	0.9	15 N, 12 N, 14 N
	DMSO		14.7	2.5		15, 17, 12
	Untreated Control		16.0	3.4		15, 14, 19, 12, 20
TA1537	HRJ Tallow	0.156 µl	11.7	0.6	1.0	12, 11, 12
		0.313 µl	14.7	1.2	1.3	14, 14, 16
		0.625 µl	10.0	2.0	0.9	12, 10, 8
		1.25 µl	14.3	4.0	1.3	12, 19, 12
		2.5 µl	18.3	1.5	1.6	20 N, 17 N, 18 N
		5 µl	12.3	2.3	1.1	11 N, 15 N, 11 N
	DMSO		11.3	0.6		12, 11, 11
	Untreated Control		12.6	3.0		12, 18, 11, 11, 11
TA98	HRJ Tallow	0.156 µl	24.0	5.2	0.8	18, 27, 27
		0.313 µl	21.0	4.6	0.7	22, 25, 16
		0.625 µl	22.3	0.6	0.7	22, 23, 22
		1.25 µl	25.0	2.6	0.8	22, 26, 27
		2.5 µl	28.3	2.1	0.9	29 N, 30 N, 26 N
		5 µl	17.3	2.1	0.5	15 N, 19 N, 18 N
	DMSO		32.0	1.7		33, 33, 30
	Untreated Control		29.4	4.3		25, 35, 31, 31, 25
TA100	HRJ Tallow	0.156 µl	129.7	3.8	0.9	134, 127, 128
		0.313 µl	134.3	3.5	0.9	131, 134, 138
		0.625 µl	128.7	4.9	0.9	132, 131, 123
		1.25 µl	128.0	4.4	0.9	131, 123, 130
		2.5 µl	118.3	4.9	0.8	124 N, 116 N, 115 N
		5 µl	129.0	7.9	0.9	138 N, 123 N, 126 N
	DMSO		141.7	0.6		141, 142, 142
	Untreated Control		128.2	8.9		121, 141, 128, 132, 119
WP2uvrA	HRJ Tallow	0.156 µl	19.0	1.0	0.5	20, 18, 19
		0.313 µl	22.0	3.5	0.6	26, 20, 20
		0.625 µl	23.7	1.2	0.6	25, 23, 23
		1.25 µl	17.3	2.5	0.5	20, 17, 15
		2.5 µl	23.0	1.0	0.6	23 N, 24 N, 22 N
		5 µl	25.3	1.2	0.7	24 N, 26 N, 26 N
	DMSO		38.3	2.3		41, 37, 37
	Untreated Control		40.2	6.3		38, 40, 46, 31, 46
Key to Plate Postfix Codes						
N      Normal background lawn						

**Table 4 (concluded)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**1<sup>st</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Tallow  
Experiment: G371-11 1st Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-11  
Date Plated: 3/1/2011  
Date Counted: 3/4/2011

With metabolic activation (5% S9)						
Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1535	HRJ Tallow	0.156 µl	16.0	2.6	1.0	19, 14, 15
		0.313 µl	34.0	1.0	2.2	35, 34, 33
		0.625 µl	21.7	2.9	1.4	20, 25, 20
		1.25 µl	20.3	2.3	1.3	19, 23, 19
		2.5 µl	16.0	2.6	1.0	15 N, 14 N, 19 N
		5 µl	16.0	2.0	1.0	18 N, 16 N, 14 N
	DMSO		15.7	3.2		17, 18, 12
TA1537	HRJ Tallow	0.156 µl	9.3	1.2	0.7	10, 8, 10
		0.313 µl	8.3	1.5	0.6	10, 8, 7
		0.625 µl	9.7	1.5	0.7	10, 8, 11
		1.25 µl	8.3	1.5	0.6	10, 8, 7
		2.5 µl	12.7	2.1	1.0	11 N, 12 N, 15 N
		5 µl	10.0	2.0	0.8	10 N, 12 N, 8 N
	DMSO		13.3	2.1		14, 15, 11
TA98	HRJ Tallow	0.156 µl	29.7	1.2	0.7	29, 29, 31
		0.313 µl	29.3	0.6	0.7	29, 30, 29
		0.625 µl	35.0	2.6	0.9	38, 33, 34
		1.25 µl	37.3	0.6	0.9	37, 37, 38
		2.5 µl	30.7	2.9	0.8	34 N, 29 N, 29 N
		5 µl	31.0	1.7	0.8	33 N, 30 N, 30 N
	DMSO		39.7	5.1		44, 34, 41
TA100	HRJ Tallow	0.156 µl	146.0	3.6	0.9	145, 150, 143
		0.313 µl	149.0	6.1	0.9	146, 156, 145
		0.625 µl	147.3	7.5	0.9	156, 143, 143
		1.25 µl	144.3	3.1	0.9	141, 145, 147
		2.5 µl	149.0	1.7	0.9	150 N, 150 N, 147 N
		5 µl	133.3	5.7	0.8	127 N, 138 N, 135 N
	DMSO		157.0	4.0		153, 157, 161
WP2uvrA	HRJ Tallow	0.156 µl	29.7	0.6	1.0	30, 29, 30
		0.313 µl	34.7	0.6	1.1	35, 34, 35
		0.625 µl	28.3	2.3	0.9	31, 27, 27
		1.25 µl	18.7	1.2	0.6	20, 18, 18
		2.5 µl	30.0	1.7	1.0	31 N, 31 N, 28 N
		5 µl	27.7	2.1	0.9	27 N, 30 N, 26 N
	DMSO		31.0	2.0		33, 29, 31

Key to Plate Postfix Codes

N Normal background lawn

**Table 5**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**2<sup>nd</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Positive Controls  
Experiment: G371-11 2nd Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-1  
Date Plated: 3/16/2011  
Date Counted: 3/18/2011

Without metabolic activation						
Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1535	SA	5 µg	927.0	87.2	67.8	987, 827, 967
TA1537	9AA	50 µg	238.3	28.7	26.5	242, 208, 265
TA98	2NF	5 µg	759.0	41.7	43.0	780, 786, 711
TA100	SA	5 µg	803.0	15.9	6.8	791, 797, 821
WP2uvrA	4NQO	2.5 µg	3638.7	195.6	114.9	3854, 3590, 3472
TA1535	DMSO	--	7.0	1.0	0.5	6, 7, 8
TA1537	DMSO	--	10.3	0.6	1.1	10, 10, 11
TA98	DMSO	--	12.7	3.1	0.7	12, 16, 10
TA100	DMSO	--	109.7	7.6	0.9	103, 108, 118
WP2uvrA	DMSO	--	24.7	2.5	0.8	22, 25, 27
With metabolic activation (10% S9)						
Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1535	2AN (10% S9)	4 µg	194.3	10.4	12.4	191, 186, 206
TA1537	2AN (10% S9)	4 µg	457.3	49.1	36.1	420, 439, 513
TA98	2AN (10% S9)	2 µg	849.3	35.9	28.6	873, 808, 867
TA100	2AN (10% S9)	2 µg	1063.7	58.3	8.5	1131, 1028, 1032
WP2uvrA	2AN (10% S9)	20 µg	320.7	6.8	8.3	326, 323, 313
TA1535	DMSO (+S9)	--	9.3	2.1	0.6	11, 7, 10
TA1537	DMSO (+S9)	--	14.3	3.2	1.1	18, 13, 12
TA98	DMSO (+S9)	--	32.3	4.6	1.1	27, 35, 35
TA100	DMSO (+S9)	--	129.0	0.0	1.0	129, 129, 129
WP2uvrA	DMSO (+S9)	--	34.7	2.1	0.9	33, 37, 34
Key to Positive Controls			Key to Plate Postfix Codes			
SA	Sodium Azide		N	Normal background lawn		
9AA	9-Aminoacridine hydrochloride					
2NF	2-Nitrofluorene					
4NQO	4-Nitroquinoline N-oxide					
DMSO	Dimethyl Sulfoxide					
2AN (10% S9)	2-Aminoanthracene (10% S9)					
DMSO (+S9)	Dimethyl Sulfoxide +S9					

**Table 5 (continued)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**

**2<sup>nd</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Camelina  
Experiment: G371-11 2nd Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-11  
Date Plated: 3/16/2011  
Date Counted: 3/18/2011

Strain	Compound	Dose level per plate	Without metabolic activation			Individual revertant colony counts
			Mean revertants per plate	Standard Deviation	Ratio treated / solvent	
TA1535	HRJ Camelina	0.156 µl	12.7	2.9	0.9	11, 11, 16
		0.313 µl	14.7	3.1	1.1	12, 18, 14
		0.625 µl	10.7	1.2	0.8	12, 10, 10
		1.25 µl	12.7	1.2	0.9	14, 12, 12
		2.5 µl	10.7	3.1	0.8	14, 10, 8
		5 µl	13.3	2.3	1.0	12 N, 12 N, 16 N
	DMSO		13.7	1.5		12, 14, 15
	Untreated Control		5.4	0.9		5, 5, 7, 5, 5
TA1537	HRJ Camelina	0.156 µl	14.3	0.6	1.6	14, 14, 15
		0.313 µl	11.7	1.5	1.3	10, 13, 12
		0.625 µl	11.7	2.9	1.3	10, 15, 10
		1.25 µl	15.3	2.1	1.7	13, 17, 16
		2.5 µl	15.3	1.5	1.7	14, 17, 15
		5 µl	11.0	3.6	1.2	15 N, 10 N, 8 N
	DMSO		9.0	1.7		10, 7, 10
	Untreated Control		9.2	3.1		12, 5, 10, 7, 12
TA98	HRJ Camelina	0.156 µl	25.0	4.6	1.4	20, 26, 29
		0.313 µl	27.0	2.6	1.5	30, 26, 25
		0.625 µl	25.7	0.6	1.5	26, 26, 25
		1.25 µl	21.0	3.6	1.2	20, 18, 25
		2.5 µl	21.7	1.5	1.2	22, 20, 23
		5 µl	18.7	3.5	1.1	22 N, 19 N, 15 N
	DMSO		17.7	2.5		15, 20, 18
	Untreated Control		34.0	0.7		35, 34, 33, 34, 34
TA100	HRJ Camelina	0.156 µl	135.0	1.0	1.2	136, 134, 135
		0.313 µl	119.7	4.0	1.0	119, 124, 116
		0.625 µl	108.7	3.1	0.9	108, 112, 106
		1.25 µl	98.0	6.2	0.8	105, 93, 96
		2.5 µl	88.3	3.2	0.8	87, 86, 92
		5 µl	82.3	2.3	0.7	85 N, 81 N, 81 N
	DMSO		117.3	2.1		119, 115, 118
	Untreated Control		96.8	6.0		91, 105, 101, 92, 95
WP2uvrA	HRJ Camelina	0.156 µl	30.0	1.7	0.9	31, 28, 31
		0.313 µl	32.7	3.8	1.0	31, 37, 30
		0.625 µl	32.7	4.6	1.0	38, 30, 30
		1.25 µl	30.0	4.6	0.9	29, 26, 35
		2.5 µl	27.3	1.5	0.9	29, 27, 26
		5 µl	26.3	4.2	0.8	31 N, 23 N, 25 N
	DMSO		31.7	2.3		33, 29, 33
	Untreated Control		29.8	5.0		22, 33, 30, 29, 35
						Key to Plate Postfix Codes
						N Normal background lawn



**Table 5 (continued)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**2<sup>nd</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Camelina  
Experiment: G371-11 2nd Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-11 Camelina  
Date Plated: 3/16/2011  
Date Counted: 3/18/2011

Strain	Compound	Dose level per plate	With metabolic activation (10% S9)			Individual revertant colony counts
			Mean revertants per plate	Standard Deviation	Ratio treated / solvent	
TA1535	HRJ Camelina	0.156 µl	10.3	2.1	0.7	12, 8, 11
		0.313 µl	11.3	1.2	0.7	12, 10, 12
		0.625 µl	6.0	1.7	0.4	7, 7, 4
		1.25 µl	7.7	2.1	0.5	6, 7, 10
		2.5 µl	11.3	0.6	0.7	12, 11, 11
		5 µl	13.0	2.6	0.8	15 N, 10 N, 14 N
	DMSO		15.7	2.1		14, 18, 15
TA1537	HRJ Camelina	0.156 µl	13.0	2.6	1.0	15, 10, 14
		0.313 µl	12.3	2.5	1.0	15, 12, 10
		0.625 µl	12.3	3.2	1.0	10, 16, 11
		1.25 µl	19.7	2.5	1.6	22, 17, 20
		2.5 µl	13.3	3.1	1.1	14, 16, 10
		5 µl	10.0	2.0	0.8	10 N, 12 N, 8 N
	DMSO		12.7	1.2		12, 14, 12
TA98	HRJ Camelina	0.156 µl	32.3	2.9	1.1	34, 29, 34
		0.313 µl	39.7	6.1	1.3	45, 41, 33
		0.625 µl	31.0	5.6	1.0	26, 30, 37
		1.25 µl	28.3	5.0	1.0	23, 29, 33
		2.5 µl	30.0	0.0	1.0	30, 30, 30
		5 µl	33.7	2.5	1.1	36 N, 34 N, 31 N
	DMSO		29.7	2.5		30, 32, 27
TA100	HRJ Camelina	0.156 µl	113.7	11.0	0.9	110, 105, 126
		0.313 µl	118.3	3.2	0.9	116, 122, 117
		0.625 µl	114.3	10.1	0.9	108, 109, 126
		1.25 µl	110.0	7.5	0.9	117, 111, 102
		2.5 µl	107.7	3.8	0.9	112, 106, 105
		5 µl	98.7	7.1	0.8	100 N, 105 N, 91 N
	DMSO		125.3	5.5		125, 131, 120
WP2uvrA	HRJ Camelina	0.156 µl	32.0	4.6	0.8	33, 36, 27
		0.313 µl	33.3	5.8	0.9	30, 30, 40
		0.625 µl	32.0	4.4	0.8	35, 27, 34
		1.25 µl	30.3	0.6	0.8	31, 30, 30
		2.5 µl	32.0	3.6	0.8	31, 29, 36
		5 µl	36.0	3.6	0.9	40 N, 35 N, 33 N
	DMSO		38.7	2.9		37, 42, 37
						<b>Key to Plate Postfix Codes</b>
						N      Normal background lawn



**Table 5 (continued)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**2<sup>nd</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-11 Tallow  
Experiment: G371-11 2nd Mutagenicity  
Assay Conditions: Plate incorporation assay

Study Code: G371-11  
Date Plated: 3/16/2011  
Date Counted: 3/18/2011

Strain	Compound	Dose level per plate	Without metabolic activation			Individual revertant colony counts
			Mean revertants per plate	Standard Deviation	Ratio treated / solvent	
TA1535	HRJ Tallow	0.156 µl	10.7	2.3	0.8	12, 12, 8
		0.313 µl	8.0	3.0	0.6	11, 8, 5
		0.625 µl	9.0	1.7	0.7	11, 8, 8
		1.25 µl	6.0	1.0	0.4	6, 7, 5
		2.5 µl	7.0	1.7	0.5	8, 8, 5
		5 µl	7.0	1.0	0.5	7 N, 8 N, 6 N
	DMSO		13.7	1.5		12, 14, 15
	Untreated Control		5.4	0.9		5, 5, 7, 5, 5
TA1537	HRJ Tallow	0.156 µl	8.0	2.6	0.9	5, 9, 10
		0.313 µl	10.0	1.0	1.1	9, 11, 10
		0.625 µl	10.0	2.0	1.1	12, 8, 10
		1.25 µl	7.3	2.5	0.8	10, 7, 5
		2.5 µl	7.7	1.5	0.9	8, 6, 9
		5 µl	6.0	1.7	0.7	5 N, 5 N, 8 N
	DMSO		9.0	1.7		10, 7, 10
	Untreated Control		9.2	3.1		12, 5, 10, 7, 12
TA98	HRJ Tallow	0.156 µl	19.7	2.1	1.1	19, 18, 22
		0.313 µl	21.3	4.0	1.2	26, 19, 19
		0.625 µl	16.3	4.7	0.9	18, 11, 20
		1.25 µl	18.3	5.8	1.0	15, 15, 25
		2.5 µl	21.0	5.6	1.2	26, 22, 15
		5 µl	15.3	0.6	0.9	15 N, 16 N, 15 N
	DMSO		17.7	2.5		15, 20, 18
	Untreated Control		34.0	0.7		35, 34, 33, 34, 34
TA100	HRJ Tallow	0.156 µl	139.3	5.0	1.2	134, 144, 140
		0.313 µl	127.0	6.0	1.1	121, 127, 133
		0.625 µl	129.7	8.3	1.1	123, 127, 139
		1.25 µl	115.0	4.4	1.0	112, 113, 120
		2.5 µl	122.7	8.1	1.0	119, 117, 132
		5 µl	133.7	8.4	1.1	138 N, 124 N, 139 N
	DMSO		117.3	2.1		119, 115, 118
	Untreated Control		96.8	6.0		91, 105, 101, 92, 95
WP2uvrA	HRJ Tallow	0.156 µl	24.0	2.0	0.8	26, 22, 24
		0.313 µl	23.0	1.7	0.7	22, 22, 25
		0.625 µl	20.7	1.5	0.7	19, 21, 22
		1.25 µl	25.0	3.6	0.8	24, 22, 29
		2.5 µl	28.3	4.0	0.9	26, 26, 33
		5 µl	27.3	0.6	0.9	27 N, 28 N, 27 N
	DMSO		31.7	2.3		33, 29, 33
	Untreated Control		29.8	5.0		22, 33, 30, 29, 35
						Key to Plate Postfix Codes
						N Normal background lawn

**Table 5 (concluded)**  
**INDIVIDUAL AND MEAN PLATE COUNTS**  
**2<sup>nd</sup> MUTAGENICITY EXPERIMENT WITH TWO HRJ FUELS**

Study Name: G371-1 Tallow  
 Experiment: G371-11 2nd Mutagenicity  
 Assay Conditions: Plate incorporation assay

Study Code: G371-11  
 Date Plated: 3/16/2011  
 Date Counted: 3/18/2011

With metabolic activation (10% S9)						
Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1535	HRJ Tallow	0.156 µl	11.3	1.5	0.7	13, 11, 10
		0.313 µl	14.3	3.1	0.9	15, 17, 11
		0.625 µl	13.3	0.6	0.9	13, 13, 14
		1.25 µl	15.3	2.3	1.0	18, 14, 14
		2.5 µl	13.7	2.3	0.9	11, 15, 15
		5 µl	15.3	1.5	1.0	17 N, 14 N, 15 N
	DMSO		15.7	2.1		14, 18, 15
TA1537	HRJ Tallow	0.156 µl	12.0	2.0	0.9	10, 12, 14
		0.313 µl	11.0	3.6	0.9	10, 8, 15
		0.625 µl	10.0	0.0	0.8	10, 10, 10
		1.25 µl	12.0	3.5	0.9	14, 14, 8
		2.5 µl	9.7	3.8	0.8	14, 8, 7
		5 µl	10.3	2.9	0.8	12 N, 12 N, 7 N
	DMSO		12.7	1.2		12, 14, 12
TA98	HRJ Tallow	0.156 µl	31.7	2.1	1.1	34, 31, 30
		0.313 µl	27.0	3.6	0.9	24, 26, 31
		0.625 µl	31.0	1.7	1.0	30, 33, 30
		1.25 µl	26.3	1.5	0.9	25, 28, 26
		2.5 µl	28.7	2.5	1.0	26, 31, 29
		5 µl	29.7	2.3	1.0	31 N, 31 N, 27 N
	DMSO		29.7	2.5		30, 32, 27
TA100	HRJ Tallow	0.156 µl	154.0	1.7	1.2	152, 155, 155
		0.313 µl	145.7	0.6	1.2	146, 146, 145
		0.625 µl	156.3	7.8	1.2	150, 165, 154
		1.25 µl	154.7	7.1	1.2	147, 156, 161
		2.5 µl	158.7	8.6	1.3	168, 157, 151
		5 µl	156.3	4.9	1.2	153 N, 154 N, 162 N
	DMSO		125.3	5.5		125, 131, 120
WP2uvrA	HRJ Tallow	0.156 µl	25.0	2.0	0.6	27, 25, 23
		0.313 µl	32.0	1.7	0.8	33, 30, 33
		0.625 µl	32.3	4.6	0.8	27, 35, 35
		1.25 µl	29.3	2.9	0.8	31, 31, 26
		2.5 µl	28.0	2.6	0.7	29, 25, 30
		5 µl	28.0	1.0	0.7	29 N, 27 N, 28 N
	DMSO		38.7	2.9		37, 42, 37
Key to Plate Postfix Codes						
N Normal background lawn						

## APPENDIX H. HISTORICAL VALUES FOR SPONTANEOUS REVERTANTS AND POSITIVE CONTROLS

Historical data include GLP studies conducted at SRI International from 1/05 to 3/10.

<u>Strain</u>	<u>Spontaneous Revertants</u>
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TA1535	5 - 35
TA1537	1 - 20
TA98	10 - 45
TA100	90 - 210
WP2 <i>uvrA</i>	10 – 50

<u>Strain</u>	<u>Positive Control</u>	<u>S9 (%)</u>	<u>Dose/Plate</u>	<u>Range</u>
TA1535	sodium azide	0	5 µg	780 - 2680
TA1537	9-aminoacridine	0	50 µg	108 - 800
TA98	2-nitrofluorene	0	5 µg	640 - 2790
TA100	sodium azide	0	5 µg	860 - 2630
WP2 <i>uvrA</i>	4-Nitroquinoline-N-oxide	0	2.5 µg	1285 – 4511
TA1535	2-anthramine	5/10	4 µg	215-652/180-500
TA1537	2-anthramine	5/10	4 µg	265-920/225-710
TA98	2-anthramine	5/10	2 µg	865-3905/805-2790
TA100	2-anthramine	5/10	2 µg	1065-4800/1005-3085
WP2 <i>uvrA</i>	2-anthramine	5/10	20 µg	225-920/140-775

## LIST OF ACRONYMS

°C	degrees Celsius
µg	microgram
µL	microliter
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AMS	Archive Management System
cm	centimeter
CoFA	Certificate of Analysis
DMSO	dimethyl sulfoxide
DTIC	Defense Technical Information Center
EPA	Environmental Protection Agency
F-T	Fischer-Tropsch
g	gram
GLP	Good Laboratory Practices
HEFA	hydroprocessed esters and fatty acids
HEFA-C	HEFA-Camelina
HEFA-F	HEFA-Animal fats and oils
HEFA-T	HEFA-Tallow
HJF	Henry M. Jackson Foundation for the Advancement of Military Medicine
HRJ	hydrotreated renewable jet
kg	kilogram
M	molar
MA	metabolic activation
mg	milligram
min	minutes
mL	milliliter
mm	millimeter
NCIMB	National Collection of Industrial and Marine Bacteria
NZW	New Zealand White
OECD	Organisation for Economic Cooperation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
PDII	Primary Dermal Irritation Index
rpm	rotations per minute
sec	seconds
SOP	standard operating procedure
UV	ultraviolet
v/v	volume/volume
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